Investigation of cell cycle status in patients with acute myeloid leukaemia

Rob Samuel Sellar

UCL

A thesis submitted for the degree of Doctor of Philosophy

2017

DECLARATION

I, Rob Samuel Sellar, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

Signed:

Date: 28th January 2017

ABSTRACT

Acute myeloid leukaemia (AML) is a biologically and clinically heterogeneous disease. Studies investigating heterogeneity in cell cycle status and response to treatment have been inconsistent, did not take into account the molecular features of the disease, and used blasts derived from aspirates (BMA) or peripheral blood (PB). The work in this thesis used the expression of the DNA replication licensing factors MCM2 (positive in non-G_0 cells) geminin (positive in S/G_2/M phase) in combination with MIB-1 (actively cycling cells) as assessed by immunohistochemistry and immunocytochemistry to define the cell cycle status of the blasts from PB, BMA, and biopsies (BMT). It shows that the majority of blasts in PB exist in a G_1-arrested state and these blasts cause results derived from BMA or PB to significantly underestimate disease proliferation. Further experiments using flow cytometry and RNA sequencing support these conclusions and demonstrate that accurate assessment of cell cycle status in patients requires the use of BMT. A cohort of 181 patients with AML and available BMT for the assessment of DNA replication licensing factors was identified and defined for clinical, cytogenetic, and molecular features, and clinical outcome. The features of this cohort were consistent with those from large clinical trials. Increased expression of geminin and higher geminin/MIB-1 ratios (increased speed of cycling) were associated with *NPM1* mutations and improved response to induction therapy in both univariate and multivariate analysis. Patients with lower geminin/MIB-1 ratios had increased rates of relapse and, in a landmark analysis from the second cycle of treatment, a trend towards inferior overall survival. These results suggest a mechanism for the improved response and decreased relapse risk seen in patients with *NPM1* mutations and the potential clinical utility of examining DNA replication licensing factors.

ACKNOWLEDGEMENTS

I would like to thank Professor David Linch for his expert and thoughtful guidance, support, and mentorship but also for giving me the freedom and confidence to pursue ideas. Professor Rosemary Gale deserves special thanks for the training she has offered over the years both in the lab and in my writing and despite the frustrations I must have given. Our differing opinions on the use of the comma may never be resolved. In terms of experimental advice sincere thanks are offered to Professor Asim Khwaja. Professor Karl Peggs was not involved with the project *per se* but has been a fantastic scientific and career mentor who has always given his time and focus. In the department of pathology Professor Gareth Williams and Dr Marco Loddo were instrumental in the initial stages of the project. Dr Kai Stoeber has continued to offer advice on all aspects of cell cycle analysis and research. Dr Teresa Marafioti provided expert advice on sample processing. In the department of genomics, Dr Tony Brooks and Dr Mike Hubank deserve thanks for their advice and assistance with the processing and analysis of a RNAseq data.

The department of haematology at UCL was a fantastic place to work and to learn. Friends and colleagues deserving of special thanks and who helped with science or post work discussion and beer include Chris Allen, Sarah Jenkinson, Koremu Meja, Maciej Garbowski, Simon Brooks, Marc Davies, Brad Smith, Marco Della Peruta, Steven Tinsley, Maria Virgilio, Beth Payne, and Marc Mansour.

I would like to extend my thanks to the patients who generously and willingly donated samples for this research.

Finally, I would like to thank my family. My parents for their encouragement in pursuing science, and my father for inspiring scientific aspirations even though I did not eventually focus on yeasts and brewing. Most importantly, my wife Emma has endured this thesis on both sides of the Atlantic and has given me more support than I deserve. She is now free to follow her own ambitions on the polo field. To my children, Lottie and Hamish, I owe you more ice-creams and trips to the park than I can count.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

COMMONLY USED ABBREVIATIONS

AML	Acute myeloid leukaemia
APL	Acute promyelocytic leukaemia
Ara-C	Cytarabine arabinoside
ATO	Arsenic Trioxide
ATRA	*All-trans*-retinoic acid
BM	Bone marrow
BMA	Bone marrow aspirate
BMT	Bone marrow biopsy (trephine)
bp	Base pairs
CBF	Core binding factor
cDNA	Complementary DNA
CEBPA	CCAAT-enhancer binding protein α
CIR	Cumulative incidence of relapse
CN-AML	Cytogenetically normal AML
CR	Complete remission
DDW	Double distilled water
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
EFS	Event-free survival
FAB	French-American-British
FLT3	Fms-like tyrosine kinase-3
$FLT3^{ITD}$	*FLT3* with an internal tandem duplication
$FLT3^{WT}$	*FLT3* wild-type (no internal tandem duplication)
FPKM	Fragments Per Kilobase of transcript per Million mapped read
G-CSF	Granulocyte colony-stimulating factor
GVHD	Graft-versus-host disease
GO	Gemtuzumab Ozogamicin
GVL	Graft-versus-leukaemia
HR	Hazard ratio
HSCT	Allogeneic stem cell transplantation
ID	Induction death
ITD	Internal tandem duplication

MDR-1	Multi-drug resistant protein
MDS	Myelodysplasia
MK	Monosomal karytoype
MLL	Myeloid/lymphoid leukaemia
MRC	Medical Research Council, UK
MRD	Minimal Residual Disease
NK	Normal karyotype
NPM1	Nucleophosmin 1
$NPM1^{MUT}$	Nucleophosmin 1 mutation
$NPM1^{WT}$	Nucleophosmin 1wild-type
NRM	Non-relapse mortality
ORC	Origin recognition complex
OS	Overall survival
PB	Peripheral blood
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidum Idodide
Pre-RC	Pre-replicative complex
RFS	Relapse-free survival
RNA	Ribonucleic acid
RR	Relapse rate
TKD	Tyrosine kinase domain
WCC	White blood cell count
WHO	World Health Organization
WT	Wild-type

CHAPTER 1. INTRODUCTION

1.1 Introduction

Acute myeloid leukaemia (AML) is an aggressive clonal disorder of haematopoietic stem or progenitor cells, the defining feature of which is aberrant differentiation and excess proliferation leading to the accumulation of immature myeloid cells in the bone marrow. This is accompanied by a marked reduction of normal haematopoiesis resulting in bone marrow failure.

AML displays considerable biological heterogeneity such that it may be better considered as a collection of malignancies rather than a single disease entity. This biological heterogeneity is reflected in significant differences in both presenting features and clinical outcome between patients. While some patients are cured, the majority who attain remission subsequently relapse. Others are refractory to treatment and succumb rapidly to their disease. Despite this heterogeneity, with the exception of acute promyelocytic leukaemia (APL), patients who are fit enough receive a similar initial induction treatment aimed at achieving complete remission (CR). The combination of an anthracycline with cytarabine arabinoside (Ara-C) has been the cornerstone of induction treatment for over 30 years. Although there have been improvements in outcome over this time, these have largely been the result of advances in supportive care. The use of additional drugs has failed to show marked or consistent benefit. Those studies that have shown improvements in outcome have tended to demonstrate this for those patients who would already be predicted to do better (Fernandez *et al*, 2009; Burnett *et al*, 2011). Patients with the poorest risk disease have seen little benefit. There is a clear need for better understanding of disease biology to aid disease stratification and improve treatment.

The work presented in this thesis examines inter-patient heterogeneity in cell cycle status in the context of the demographics of patients with AML and the clinical, cytogenetic, and molecular features of their disease. In addition, it examines the relationship between cell cycle status, the response to treatment, and survival. This introductory chapter will outline the current understanding of diagnosis, prognosis, pathogenesis and treatment of AML, and highlight the need and rationale for both

improved risk stratification to guide treatment and better understanding of the factors associated with response to treatment. It will then outline the rationale for studying the cell cycle in patients with AML to assist with these aims.

1.2 Overview of AML

1.2.1 Clinical presentation

The accumulation of immature myeloid cells seen within the bone marrow of patients with AML occurs as the leukaemic cells outcompete their normal counterparts and suppress normal haematopoiesis (Miraki-Moud *et al*, 2013). Typical clinical features of bone marrow failure result and may include fatigue and dyspnoea from anaemia, bleeding from thrombocytopenia, and infections from true or functional neutropenia. A bleeding diathesis may also result from disseminated intravascular coagulation, which is associated with, though not restricted to, patients with APL. Patients may also present with leukocytosis and leukostasis. In addition, organs including skin and central nervous system may be affected by leukaemic infiltration.

1.2.2 Incidence

AML is the commonest acute leukaemia in adults, with an annual incidence of 3.4 cases per 100,000 adults in the UK and a total of 2,856 new cases diagnosed in the UK in the year 2012 (figures from Cancer Research UK). Incidence increases with age, rising from 1.8 per 100,000 in young adults up to 17 per 100,000 in individuals ≥60 years of age (Deschler & Lubbert, 2006). Almost three quarters of cases are diagnosed in those ≥60 years of age.

1.2.3 Predisposing factors

Although the majority of new patients with AML have idiopathic (*de novo*) disease, there are recognised predisposing factors and diseases seen in clinical practice, some of which represent distinct entities in the World Health Organisation (WHO) classification of AML described later in this chapter (Arber *et al*, 2016).

1.2.3.1 Therapy-related AML

Two main types of chemotherapy-related acute myeloid leukaemia exist. Drugs that target topoisomerase II, such as anthracyclines (e.g. daunorubicin) and epidophyllotoxins (e.g. etoposide), can cause patients to develop a rapidly proliferative monoblastic disease with cytogenetic abnormalities at the *MLL* gene locus at chromosome 11q23 within 2 years of treatment. More commonly treatment-related AML follows the use of alkylating agents (e.g. melphalan and cyclophosphamide), with disease occurring 5-6 years after exposure to drug. This is characterised by a myelodysplastic prodrome with complex karytoype and typically deletions in chromosomes 5 and/or 7. Therapy-related AML may also develop following treatment with radiotherapy.

1.2.3.2 Secondary AML

Transformation to AML may occur following clonal evolution from acquired syndromes such as myelodysplasia (MDS), chronic myeloid leukaemia (CML), and the myeloproliferative neoplasms polycythaemia vera (PV), essential thrombocythaemia (ET) and myelofibrosis (MF).

1.2.3.3 Familial AML

A variety of inherited genetic defects may predispose to the development of AML. These include Shwachmann-Diamond syndrome, Fanconi anaemia, and ataxia telangiectasia. In addition, a number of germline mutations in families have been associated with the development of myeloid disorders and ultimately AML, including mutations of *RUNX1*, *CEBPA* and *GATA2,* all genes encoding key transcription factors involved in haematopoiesis (Owen *et al*, 2008; Hahn *et al*, 2011)

1.2.3.4 AML related to chromosomal disorders

Many of the congenital disorders of chromosome copy number predispose to the development of leukaemia, including AML with trisomy 21 that is classically associated with the development of AML with an M7 phenotype (see section 1.3.1 below).

1.3 Diagnosis and classification

1.3.1 FAB classification

The first comprehensive classification of AML was the French-American-British (FAB) system (Bennett *et al*, 1976; Bennett *et al*, 1985) that required a minimum of 30% blasts within the bone marrow compartment and stratified AML according to morphology- and cytochemistry-defined lineage type and degree of differentiation. A summary of the FAB classification is given in Table 1.1. Some FAB types are associated with particular recurrent cytogenetic abnormalities, for example, t(8;21), t(15;17) and t(16;16)/inv(16) with subtypes M2, M3 and M4Eo respectively.

Table 1.1 The French-American-British (FAB) Classification of AML.

FAB subtype	Name	Cytochemistry			Morphological criteria
		MPO	SB	NSE	
M0	AML with minimal differentiation	-	-	-	Blasts ≥30% of BM nucleated cells Blasts ≥30% of BM non-erythroid cells <3% of blasts positive for SB or MPO by light microscopy
M1	AML without maturation	+	+	-	Blasts ≥30% of BM cells Blasts ≥90% of BM non-erythroid cells ≥3% of blasts positive for SB or MPO
M2	AML with maturation	+	+	-	Blasts ≥30% of BM cells Blasts 30-89% of BM non-erythroid cells Monocytic component <20% of non-erythroid cells
M3	Acute promyelocytic leukaemia	+	+	-	Majority of infiltrate are abnormal promyelocytes
M4	Acute myelomonocytic leukaemia	+	+	+	Blasts ≥30% of BM cells Blasts ≥30% of BM non-erythroid cells Granulocytic component ≥20% of non-erythroid cells Significant monocytic component
M4(Eo)	Acute myelomonocytic leukaemia with abnormal eosinophils	+	+	+	Blasts ≥30% of BM cells Blasts ≥30% of BM non-erythroid cells Granulocytic component ≥20% of non-erythroid cells Significant monocytic component
M5	Acute monocytic leukaemia 5a: monoblastic 5b: monocytic	-	-	-	Blasts ≥30% of BM cells Blasts ≥30% of BM non-erythroid cells Monocytic component ≥80% of non-erythroid cells
M6	AML with predominant erythroid differentiation 6a erythroleukaemia 6b pure erythroid leukaemia	+	+	-	Erythroblasts ≥50% of BM nucleated cells Blasts ≥30% of BM non-erythroid cells
M7	Acute megakaryoblastic leukaemia	-	-	+	Blasts ≥30% of BM non-erythroid cells Cells express CD41, CD42b, or CD61

Abbreviations: MPO, myeloperoxidase; NSE, non-specific esterase; SB, Sudan Black

1.3.2 WHO classification

The WHO classification (Table 1.2) integrates morphological, cytogenetic, immunophenotyping and molecular data into one system and has largely superseded the FAB classification (Arber *et al*, 2016). Some important modifications include the lowering of the requisite blast count to 20% except in cases with t(8;21), t(15;17) or t(16;16)/inv(16), where the diagnosis may be made irrespective of blast percentage. It also introduces AML with mutated *NPM1* and AML with biallelic mutations in *CEBPA* as distinct entities, and AML with mutated *BCR-ABL1* or *RUNX1* as provisional entities. The WHO classification also tries to discern events leading to leukaemogenesis by including therapy-related AML as a category. Although still developing, such an approach has the advantage of trying to focus on the fundamental biology of the disease that may guide prognosis and treatment, as well as providing a more robust platform for consistent standardised diagnosis.

1.4 Molecular basis of AML

The recurrent cytogenetic and molecular lesions found in AML have also been demonstrated to be key events in leukaemogenesis. The classical two-hit model of leukaemogenesis required a haematopoietic stem/progenitor cell to have a lesion resulting in activated signalling (Class I mutation), *e.g.* mutations of *FLT3*, *KIT*, and *RAS,* in addition to a lesion altering transcription and differentiation (Class II mutation), *e.g.* the in-frame chimeric fusion genes *RUNX1/ETO*, *PML/RARα* and *CBFB/MYH11* that result from the balanced chromosomal rearrangements t(8;21), t(15;17) or t(16;16)/inv(16) respectively) (Kelly & Gilliland, 2002). However, whole genome-wide analysis of patient samples, culminating in the Cancer Genome Atlas

Table 1.2. The WHO Classification of AML

Acute myeloid leukaemia with recurrent genetic abnormalities	AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
	AML with inv(16)(p13.1;q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	APL with t(15;17)(q22;q12); *PML-RARA*
	AML with t(9;11)(p22;q23); *MLLT3-KMT2A*
	AML with t(6;9)(p23;q34); *DEK-NUP214*
	AML with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); *GATA2,MECOM*
	AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
	AML with *BCR-ABL1* (provisional entity)
	AML with mutated *NPM1*
	AML with mutated *CEBPA*
	AML with mutated *RUNX1* (provisional entity)
Acute myeloid leukaemia with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
Acute myeloid leukaemia, not otherwise specified	AML with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukaemia
	Acute monoblastic/monocytic leukaemia
	Acute erythroid leukaemia
	Acute megakaryoblastic leukaemia
	Acute basophilic leukaemia
	Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferations related to Down's syndrome	Transient abnormal myelopoiesis
	Myeloid Leukaemia associated with Down's syndrome

Data from (Arber *et al*, 2016)

project which reported 200 AML genome sequences, has resulted in a rapid expansion in the list of genes recurrently mutated in AML and shows that this two-hit model is now too simple (Cancer Genome Atlas Research, 2013). Genetic lesions can now be

broadly characterised into 9 groups (Table 1.3), although even here there is further complexity, for example *WT1* at least partially exerts its effect through alteration in TET2 activity, thus potentially fulfilling criteria for a Class 3 and a Class 4 mutation (Rampal *et al*, 2014). It is worth noting that within this proposed system, AML with mutations in *NPM1* are still regarded as distinct class.

The genetic landscape changes markedly with age (Rubnitz & Inaba, 2012) and also with disease ontogeny. For example, the alterations found in secondary AML are distinctly different to those found in *de novo* disease (Lindsley *et al*, 2015), with a bias towards splicing factor mutations and a negative association with mutations of *NPM1*.

This co-occurrence of genetic alterations with different functional effects on haematopoietic progenitors is a multi-step process and gives rise to clonal heterogeneity with a subclonal architecture (Walter *et al*, 2012; Welch *et al*, 2012; Bochtler *et al*, 2013; Klco *et al*, 2014). The order of acquisition of the genetic lesions can be inferred from assessing the variant allele frequency/fraction (VAF), and genome-wide analysis can reveal some of the overall clonal architecture by assessing VAF in multiple genes. Whilst the order of mutations can vary between patients, a number of mutations have been well established as recurrent founder mutations. Furthermore, some of the earliest pre-leukaemic lesions, e.g. *DNMT3A* mutations, may persist in remission and yet remain compatible with long-term disease-free survival (Ploen *et al*, 2014). In addition, early lesions, e.g. *NPM1*, are in general stable in the event of relapse, suggesting that AML relapse emerges from incompletely eradicated founder clones rather than from the development of new malignant clones (Kronke *et al*, 2013).

The earliest lesions are typically involved in epigenetic modification, e.g. *DNMT3A* and *TET2*, and are also among the most frequent genetic lesions in the recently defined entity clonal haematopoiesis of indeterminate potential (CHIP) (Genovese *et al*, 2014; Jaiswal *et al*, 2014). Although clonal haematopoiesis with increasing age had been described previously by analysis of X-inactivation (Busque *et al*, 1996; Gale *et al*, 1997), recent studies show that mutations such as *DNMT3A* and *TET2* are common in the general population, increase in frequency with age, and confer an increased risk of AML (Jaiswal *et al*, 2014). Although the majority of people with CHIP never develop

a myeloid disorder, they do have an increase in all-cause mortality, with the greatest increase in death resulting from cardiovascular disease (Jaiswal *et al*, 2014).

Table 1.3 Recurrent mutations in AML according to functional group

Class	Functional Group	Examples	Approximate frequency in AML
1	Transcription factor resulting from gene fusions	t(8;21) t(16;16) t(15;17)	18%
2	Nucleophosmin 1	NPM1	27%
3	Tumour suppressor genes	TP53 WT1 PHF6	16%
4	DNA methylation-related genes	DNMT3A IDH1 IDH2 TET2	44%
5	Activated signalling genes	FLT3 KIT N-RAS K-RAS	59%
6	Chromatin modifiers	ASXL1 EZH2	30%
7	Myeloid transcription factor genes	CEBPA RUNX1	22%
8	Cohesin complex	SMC1A SMC3 RAD21 STAG1 STAG2	13%
9	Spliceosome complex	SRSF2 SF3B1 U2AF1 ZRSR2	14%

Data taken from (Meyer & Levine, 2014)

1.5 Prognostic factors in AML

Given the heterogeneity in both patient factors and disease-related factors, a key goal is to integrate this information to develop prognostic models that can predict outcome. This may allow the development of improved risk-adapted approaches to treatment, *e.g.* who should be recommended to receive an allogeneic stem cell transplant in first remission and who may be eligible for or better served by novel treatments. Furthermore, there are a variety of reasons for treatment failure including co-morbidities precluding optimal induction therapy, treatment-related early death, failure to achieve remission (primary refractory disease), and relapsed disease.

1.5.1 Patient-related factors

Increasing age is an adverse prognostic factor in AML leading to a decrease in overall survival (OS) (Appelbaum *et al*, 2006; Juliusson *et al*, 2009) and notably retains its poor prognostic impact in multivariate analysis. The large United Kingdom Medical Research Council (MRC) trials demonstrated that for patients >60 years of age who were deemed fit enough for intensive chemotherapy, the 5-year OS was only 14% (Smith *et al*, 2011). There are several high-risk features that increase with age including adverse cytogenetics, higher rates of secondary AML, and intrinsic chemoresistance including the expression of the multidrug resistance protein (MDR-1) (Slovak *et al*, 2000; Appelbaum *et al*, 2006; Erba, 2007; Smith *et al*, 2011). In addition, performance status, although associated with increasing age, is independently associated with poorer outcomes and is also a predictor of early treatment-related death (Walter *et al*, 2011).

1.5.2 Disease-related factors

1.5.2.1 Clinical factors

Analysis of the MRC AML 10 and 12 trials revealed a number of simple measurable clinical parameters with prognostic relevance. In addition to age, factors at diagnosis

predicting for inferior outcome include secondary AML and a high peripheral blood white cell count. The response to initial treatment also predicts outcome. In the AML 12 trial, >15% blasts in the marrow after first cycle of induction treatment was indicative of a poor prognosis compared to patients with 5-15% blasts or <5% blasts (5-year OS, 26%, 44% and 56%, respectively). This is consistent with the results of the earlier MRC AML 10 trial where patients with >15% blasts had 5-year OS of 23% compared with 53% for patients with <5% blasts.

In time, greater stratification may be possible with the use of minimal residual disease (MRD) assessment by flow or molecular analysis to more accurately assess the burden of residual disease and with greater sensitivity (Freeman *et al*, 2013; Grimwade & Freeman, 2014; Chen *et al*, 2015). Suitable examples that have been studied include assessment of fusion transcript levels in patients with core-binding factor leukaemias [t(8;21) or t(16;16)/inv(16)] and APML [t(15;17)] (Yin *et al*, 2012). *NPM1* also has the potential to be a clinically useful MRD marker in that it should be essentially undetectable in remission but is rarely absent at relapse (Kronke *et al*, 2013; Ivey *et al*, 2016). However, clonal evolution of the disease limits the number of molecular markers that can be usefully employed for this purpose. Internal tandem duplications of the *FLT3* gene (*FLT3*-ITDs) are too unstable at relapse (Kottaridis *et al*, 2002) and *DNMT3A* mutations persist in remission (Ploen *et al*, 2014).

1.5.2.2 Cytogenetic factors

Data from the MRC AML 10 trial that recruited over 1600 children and adults up to age 55 and for whom karyotype data was available in 83%, revealed clear prognostic differences for different cytogenetic lesions. Patients could be defined into 3 broad groups with favourable risk cytogenetics (5-year OS, 72%), intermediate risk cytogenetics (44%), or adverse risk cytogenetics (17%) (Grimwade *et al*, 1998). This study has been updated to include patients from MRC AML 12 and MRC AML 15 and includes 5876 patients with AML aged 16-59 years (Grimwade *et al*, 2010). This study confirmed the major findings of the original study whilst refining some risk- groups (e.g. changed definition of complex karyotype from ≥5 to ≥4 cytogenetic abnormalities),

and being able to include prognostic information for some of the less frequent recurrent lesions that were present in too few numbers in the original study

Table 1.4. The MRC Classification of Cytogenetic Risk Groups

Risk group	Proportion of patients	Original MRC classification[1]	Refined MRC classification[2]
Favourable	12% 8% 3%	t(15;17) t(8;21) inv(16) or t(16;16)	t(15;17) t(8;21) inv(16) or t(16;16)
Intermediate	42% 25%	Normal Karyotype Other non-complex	Normal Karyotype Other non-complex
Adverse	10%	abn(3q) -5 or del(5q) -7 Complex (≥ 5 unrelated abnormalities)	abn(3q) excluding t(3;5) inv(3) or t(3;3) add(5q), del(5q) or -5 7, add(7q) or del(7q) t(6;11) t(10;11) t(11q23) excluding t(9;11) and t(11;19) t(9;22) -17 or abn(17p) Complex (≥ 4 unrelated abnormalities)

[1] (Grimwade *et al*, 1998); [2] (Grimwade *et al,* 2010)

to allow for definitive statements on outcome. The original and refined MRC cytogenetic classifications are shown in Table 1.4. Within each broad group there is also variation in outcome depending on the individual cytogenetic lesion. For example, patients with APL t(15;17) do better than other favourable risk patients, and patients with inv(3) do worse within the adverse risk group. Examples of 10-year OS figures for some of the most frequent recurrent cytogenetic abnormalities (or those incorporated into the WHO classification) are shown in Table 1.5.

Table 1.5 10-year overall survival according to cytogenetic lesion

Cytogenetic abnormality	**10-year overall survival (%)**
t(15;17)	81
t(8;21)	61
inv(16)	55
t(9;11)	39
t(6;9)	27
Other t(11q23)	22
-7/del(7q)	10
-5/del(5q)	6
inv(3)	3

Data from (Grimwade *et al*, 2010)

The combination of cytogenetic lesions may also be important for prognosis. For example, in the MRC data, favourable risk lesions maintain their favourable status irrespective of additional cytogenetic lesions. It has also been demonstrated outwith the MRC trials that the 'monosomal karyotype' (one monosomy with at least one additional autosomal monosomy, or with one additional chromosomal structural abnormality, excluding favourable risk lesions) has a particularly poor prognosis within the adverse risk group (4-year OS, 4%) (Breems *et al*, 2008).

Beyond the level of conventional metaphase banding analysis, cryptic chromosomal changes may be demonstrated by single-nucleotide polymorphism (SNP) arrays or by

whole genome sequencing. Although SNP arrays do not replace banding analysis or fluorescence in situ hybridisation (FISH) as they cannot demonstrate balanced translocations, they can show cryptic changes in large numbers of patients who had been labelled as normal karyotype. This is exemplified by the Cancer Genome Atlas project which detected a number of cryptic fusion genes by sequencing (Cancer Genome Atlas Research, 2013).

1.5.2.3 Integrating cytogenetic and clinical prognostic features

Data on response to first treatment and cytogenetics from the MRC AML trials was used to derive the "Wheatley score" with the aim of aiding risk stratification in future trials (Wheatley *et al*, 1999). Table 1.6 outlines this prognostic scoring system and the 5-year OS and relapse risk for patients in the different prognostic groups treated on the MRC AML 12 trial. Based on the outcome of the AML 12 and AML 15 trials this risk scoring system has been refined and updated to include the additional clinical features of patient age, sex, whether the AML is *de novo* or secondary, and the peripheral blood white cell count at diagnosis. This scoring system (Table 1.7) was used for risk stratification in the MRC AML 17 trial and to define 3 risk groups with 5-year OS of 63%, 47%, and 24% respectively.

Table 1.6 The MRC derived Wheatley score

Prognostic Risk Group	**Patients included**	**5-year OS**	**5-year RR**
Good	Any patient with t(8;21), t(16;16)/inv(16), or t(15;17) irrespective of other genetic abnormalities or bone marrow status following course 1.	76%	25%
Standard	Any patient not in good or poor risk groups	48%	52%
Poor	>15% blasts in bone marrow following course 1 *or* adverse cytogenetic lesion [-5, -7, del(5q), abn3(q), t(9;22)] *or* complex cytogenetics (≥5 abnormalities without a favourable genetic abnormality)	21%	73%

Abbreviations: OS; overall survival, RR; relapse risk

Table 1.7 The AML17 risk stratification formula

0.01325 x age (in years)
+
0.16994 x sex (1 = male, 0 = female)
+
0.2231 x diagnosis (0 = *de novo*, 1 = secondary)
+
0.65082 x cytogenetics (1 = favourable, 2 = intermediate, 3 = adverse)
+
0.19529 x status post course 1 (1 = CR, 2 = PR, 3 = no response)
+
0.00169 x WCC ($x10^9$/l)

Abbreviations: CR; complete remission, PR; partial response

1.5.2.4 Molecular markers in AML

Recurrent molecular lesions, and their clinical impact, have led to further refinement of prognostic models of AML. For example, *TP53* mutations are associated with AML with complex karyotype and have a particularly poor prognosis (Haferlach *et al*, 2008; Rucker *et al*, 2012). In the favourable prognosis cohort, mutations in *KIT* confer an adverse prognosis (Allen *et al*, 2013). However, the context in which molecular mutations have had the greatest impact is in the approximately 50% of patients with normal karyotype AML that do not have a demonstrable cytogenetic lesion using metaphase or FISH analysis.

There are numerous recurrent molecular lesions in cytogenetically normal AML (CN-AML) and some of the most frequent of these and their general prognostic implications are shown in Table 1.8. The exact prognostic impact of a mutation can relate to the mutation level (measured by allelic burden or VAF) and the presence of co-existent mutations. The prognostic implication of combinations of lesions has recently been explored in a large international study of over 1500 patients treated in

Table 1.8 Recurrent genetic mutations with prognostic significance in intermediate karyotype and cytogenetically normal AML (CN-AML)

Mutation	Frequency in CN-AML	Positive association	Negative association	Prognosis in CN-AML	Selected References
NPM1	42-62%	*FLT3*-ITD/TKD *DNMT3A* *IDH1*/2	*CEBPA* biallelic	Favourable, particularly in the absence of *FLT3*-ITD or *DNMT3A* mutation	(Schnittger *et al*, 2005; Gale *et al*, 2008; Gale *et al*, 2015)
FLT3-ITD	30-35%	*NPM1*		Adverse, particularly in the absence of *NPM1* mutation. Increasing adverse impact with increasing allelic burden	(Kottaridis *et al*, 2001; Gale *et al*, 2008; Schlenk *et al*, 2008; Linch *et al*, 2014)
DNMT3A	20-25%	*NPM1* *FLT3*-ITD	*CEBPA* biallelic	Adverse, particularly in the absence of *NPM1* mutation	(Ley *et al*, 2010; Thol *et al*, 2011; Gale *et al*, 2015)
IDH1/IDH2	30%	*NPM1* *FLT3*-ITD	*TET2* *WT1*	Controversial, likely dependent on the specific mutation and the context of co-existing mutations	(Abbas *et al*, 2010; Boissel *et al*, 2010; Green *et al*, 2010a; Marcucci *et al*, 2010; Paschka *et al*, 2010; Thol *et al*, 2010; Green *et al*, 2011)
TET2	23-27%		*IDH1/IDH2*	Controversial but may confer an adverse prognosis	(Chou *et al*, 2011; Gaidzik *et al*, 2012)
FLT3-TKD	14%	*NPM1*		Controversial, possibly favourable	(Mead *et al*, 2007; Bacher *et al*, 2008; Whitman *et al*, 2008)
CEBPA	8-19%	*FLT3*-ITD (for monoallelic *CEBPA)*	*NPM1* (for biallelic *CEBPA)*	Favourable if biallelic	(Pabst *et al*, 2009; Wouters *et al*, 2009; Dufour *et al*, 2010; Green *et al*, 2010b)

prospective clinical trials (Papaemmanuil *et al*, 2016). Amongst the most frequent and prognostically significant mutations, particularly in patients with normal karyotype, is *NPM1*, and the impact of this mutation further depends on the presence or absence of cooperating lesions, most notably a *FLT3*-ITD. The biological and prognostic impact of these mutations is discussed briefly here before being returned to in Chapter 4.

1.5.2.4.1 *NPM1* mutations

Mutations in the gene encoding nucleophosmin 1 protein (NPM1) disrupt the nucleolar localisation signal and encode a novel nuclear export signal, which leads to aberrant localisation of the protein in the cytosol. This is thought to disrupt the usual protein function that includes diverse cellular processes such as ribosome biogenesis, regulation of apoptosis, and DNA repair. Information from mutation levels suggests that *NPM1* mutations are usually heterozygous and found in all leukaemic cells of *NPM1*-mutant AML (Gale *et al*, 2008). This implies that these mutations are early events in leukaemogenesis. Although one of the commonest mutations in AML, being found in between a third and a quarter of patients, it is particularly common in CN-AML. As well as being ascribed as a distinct entity in the WHO classification system, it is a cornerstone of the European LeukemiaNet guide for prognosis in AML (Table 1.9). Although generally conferring a favourable prognosis, the impact of *NPM1* mutations may be dependent on the context of other co-existent recurrent mutations with which they are positively correlated, *e.g. DNMT3A, IDH1* and *IDH2* mutations and *FLT3*-ITD. The presence of a *FLT3*-ITD (described below), abrogates the positive impact of *NPM1* mutation.

1.5.2.4.2 *FLT3*-ITD

FLT3 is a cytokine receptor belonging to the class III family of receptor tyrosine kinases. *FLT3*-ITDs are found in approximately 25% of younger adults with AML and are more frequent in patients with a normal karyotype and patients with APL. These duplications

are always multiples of 3 base pairs (bp) and remain in-frame. The consequence of the duplication is to disrupt the negative regulatory activity of the juxtamembrane domain, cause constitutive activation of the receptor, and enhanced signalling through the downstream RAS, MAPK, and STAT5 pathways This alters the normal role of FLT3 in cell proliferation, differentiation and survival. Except in patients with APL, *FLT3*-ITD mutations are associated with poor prognosis, including lower CR rates, higher rates of relapse and decreased OS (Small, 2006), although the majority of studies have not shown decreased CR rates. There is large variation between the mutant level seen in patients. Some have mutation levels <25% of total *FLT3* alleles suggesting that not all cells carry a heterozygous mutation. Conversely, other patients have a mutational level greater than 50%, suggesting loss of the wild-type *FLT3* allele. This loss occurs by uniparental disomy and is associated with a particularly poor outcome (Gale *et al*, 2008).

Table 1.9 The European LeukemiaNet prognostic stratification of AML

Risk profile	**Subsets**
Favourable	t(8;21)(q22;q22); *RUNX1-RUNX1T1* inv(16)(p13.1q22)or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD (normal karyotype) Biallelic mutated *CEBPA* (normal karyotype)
Intermediate-I	Mutated *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	t(9;11) (p22;q23); *MLLT3-KMT2A* Cytogenetic abnormalities not classified as favourable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *GATA2-MECOM* (*EVI1*) t(6;9) (p23;q34); *DEK-NUP214* t(v;11) (v;q23); *KMT2A* rearranged -5 or del(5q); -7; abnl(17p); complex karyotype

1.6 Treatment of AML

Despite the advances in the genetic characterisation of AML and the identification of novel potential treatment targets, the mainstay of treatment for patients without APL remains induction therapy with an anthracycline, normally daunorubicin, for 3 days combined with daily Ara-C, often as a continuous infusion; the so called "3+7" approach. The aim of induction is to achieve a CR, which is commonly defined as <5% blasts in a normocellular bone marrow with normal trilineage haematopoiesis in the absence of extramedullary disease in a patient who is transfusion-independent and with a peripheral blood neutrophil count >1 $x10^9$/l and platelet count >100 $x10^9$/l. Without further treatment, disease-free survival is limited, and so patients normally proceed to consolidation treatment aimed at eliminating residual leukaemia and reducing the risk of relapse. However, despite the extensive experience with this approach, many questions about standard induction therapy remain.

1.6.1 What dose of anthracycline should be used for induction therapy?

Following evidence that higher doses of daunorubicin up to 90mg/m^2 may be beneficial, this question has been explored in several studies (Fernandez *et al*, 2009; Lowenberg *et al*, 2009). Such investigations have generally shown that this higher dose is superior to a daily dose of 45mg/m^2 for patients up to the age of 65 years. The higher dose of 90mg/m^2 in these studies was associated with improved CR rates without demonstrable delayed recovery or jeopardising of other post-remission treatments. Particular benefit was seen for patients with *NPM1,* and the results of an updated reports suggests this benefit extends to patients with *DNMT3A* or *FLT3*-ITD mutations (Luskin *et al*, 2016). However, in the UK NCRI AML 17 trial patients were randomised to receive either 60mg/m^2 or 90mg/m^2 and, following a significant increase in mortality at 60 days in the 90mg/m^2 cohort, this randomisation was terminated (Burnett *et al*, 2015a). To date no differences in OS have been reported between the two cohorts, although the report of a subgroup analysis suggests a beneficial effect of the higher dose of anthracycline on the rates of relapse and OS in patients with *FLT3*-ITD (Burnett *et al*, 2016). In a systematic review there was some evidence that idarubicin may give superior OS to

daunorubicin although the studies included predate the use of at least 60mg/m^2 in induction and one third of the studies had no survival data available to contribute to a meta-analysis (Li *et al*, 2015).

1.6.2 Should additional cytotoxics be given during induction?

Studies investigating use of additional chemotherapy agents to the standard "3+7" backbone have been generally disappointing, although occasional improvements have been reported. A trial from the Polish Adult Leukaemia Group showed improved CR rates and potentially improved OS with the addition of cladribine, with particular benefit in older patients and those with adverse risk cytogenetics (Holowiecki *et al*, 2012). Another purine analogue, fludarabine, in combination with cytarabine, G-CSF, and idarubicin (FLAG-Ida) showed no difference in OS compared with DA in the UK MRC AML 15 trial. Remissions were more frequent with FLAG-Ida, with a concomitant reduced risk or relapse, but toxicity resulted in fewer patients completing a full number of consolidation chemotherapy cycles (Burnett *et al*, 2013). The addition of other purine analogues such as clofarabine have so far failed to show benefit in trials when used as initial therapy.

1.6.3 Is there a role for Monoclonal antibodies?

Gemtuzumab ozogamicin (Mylotarg) is an anti-CD33 monoclonal antibody conjugated to the calicheamicin toxin. There are six published studies investigating the addition of this agent to induction therapy for AML. Unfortunately, the first of these trials (SWOG S0106) closed prematurely because of an increased early mortality in the GO arm (5.5% v 1.4%, P=0.01) (Petersdorf *et al*, 2013). In addition, the United States Food and Drug Administration withdrew the drug. However, subsequent studies have tended to show a benefit when GO was used as part of induction treatment, and the findings of these individual studies have been mirrored in a meta-analysis (Hills *et al*, 2014). The optimal dosing strategy has yet to be confirmed. Furthermore, benefits appear to be restricted

to patients with favourable risk disease and a proportion with intermediate risk disease. There was no benefit seen in patients with adverse risk cytogenetics.

1.6.4 Is there a role for small molecule inhibitors?

The improved understanding of the molecular lesions underlying AML have led to the development of a wealth of small molecule inhibitors. However, despite novel trial design to rapidly assess new agents such as the AML MRC "pick-a-winner" approach, progress has been limited (Hills & Burnett, 2011). Part of this may reflect the choice of target, an inability to target effectively, or selection for resistance. One of the main therapies investigated has been *FLT3* inhibitors and collectively the trials and studies have been disappointing. Even studies that have shown good initial responses and blast clearance have ultimately failed. This may reflect the fact that *FLT3* mutations are generally late events and these inhibitors will have less effect on subclones lacking the mutation. Furthermore, a recurring theme from studies is the outgrowth of *FLT3* mutants with point mutations that confer resistance to inhibitors (Daver *et al*, 2015). Other problems have included pharmacokinetic problems and incomplete inhibition of the target. Despite these issues, recent reports have shown for the first time that the addition of a *FLT3* inhibitor to standard induction therapy can improve OS for patients with mutated *FLT3* (Stone et al, 2015; Knapper et al, 2016).

1.6.5 Post-remission therapy

One of the major considerations for the treatment of a patient with AML is the optimal post-remission therapy. The broad choice is between consolidation chemotherapy with further cycles of intensive chemotherapy or allogeneic stem cell transplantation (HCST). The factors taken into consideration when deciding which approach to use, and if HSCT the conditioning to be used, include the age of the patient, the risk of relapse according to the discussed prognostic factors, and the availability of a suitable donor. In effect, this is a summation of the risks of the transplant itself in terms of

toxicity, infection and graft-versus-host disease (GVHD), and the decreased risk of relapse in terms of further anti-leukaemia treatment and the graft-versus-leukaemia (GVL) effect.

In general, an HSCT is the preferred option for younger patients with poor risk disease or intermediate risk disease without an isolated *NPM1* mutation who have a sibling donor. For patients with favourable risk disease or with intermediate risk disease and an unrelated donor only, there are arguments for using consolidation chemotherapy rather than HSCT. However, the improving survival with alternative stem cell sources other than siblings, and the increased risk of relapse seen with isolated *FLT3*-ITD (in intermediate risk disease) may persuade some clinicians towards transplant in these settings.

1.6.6 Non-intensive therapy

For patients thought unable to tolerate standard intensive treatment, either because of age or comorbidity, attenuated treatment or supportive care may be offered. Low-dose Ara-C (20mg b.d. by subcutaneous injection) is the standard attenuated treatment, though not without haematological toxicity. Furthermore, there are no clear algorithms in this patient population. Although low-dose Ara-C may induce responses in up to 20% of patients, the median OS is only 6 months, and outcome for patients is highly dependent on cytogenetic risk factors. In an MRC trial where 217 patients were randomised to receive low-dose Ara-C or best supportive care with hydroxyurea, although overall there was a survival advantage for treatment with low-dose Ara-C, this advantage was not seen in those patients with adverse risk karyotype, none of whom achieved CR (Burnett *et al*, 2007).

Hypomethylating agents such as azacitidine and decitabine have also shown promise in this setting. CR rates of 15-20% have been seen, and there is a suggestion of survival advantage over other treatment including low-dose Ara-C, albeit modest. Crucially, even in the absence of CR, both drugs may induce substantial haematological improvement (Kantarjian *et al*, 2012; Lubbert *et al*, 2012).

1.6.7 APL

The clinically and biologically distinct features of APL translate into a distinct treatment approach. In addition to sensitivity to anthracyclines, APL is a paradigm for therapies that induce differentiation of cells, and this can be achieved using either all-trans retinoic acid (ATRA) or arsenic trioxide (ATO). The combination of an anthracycline with ATRA achieves CR rates of >90% and translates to cure rates of approximately 80% (Sanz & Lo-Coco, 2011). More recently, a randomised phase 3 trial that formed part of the MRC AML17 trial showed that treating with the combination of ATRA + ATO without standard cytotoxics was feasible in both high and low risk APL patients, with CR rate of 94% and a 4-year event free survival (EFS) and OS of 91% and 93%, respectively. Interestingly, although the OS was not statistically superior to that seen with the combination of idarubicin and ATRA, the EFS was superior, which was due to a significantly decreased risk of relapse (Burnett *et al*, 2015b).

1.6.8 Treatment of relapse

Unfortunately, the majority of patients with non-APL disease relapse, and there is little data from prospective clinical trials to guide treatment post-relapse. However, the general approach is to offer reinduction chemotherapy with the aim of achieving a second remission (CR2) followed by an HSCT. There are well-established risk-factors for prognosis following relapse including older age, adverse cytogenetics at initial diagnosis, a shorter duration of first remission (<12-18 months), and previous allogeneic transplantation. Such features are associated with lower rates of achieving CR2 and of OS (Breems *et al*, 2005), and patients with unfavourable prognostic features, particularly if more than one are present, may be better served by investigational treatments or palliation.

1.7 Unresolved issues in the biology and prognosis of AML

Despite the wealth of understanding of biological and prognostic markers and available treatments, there are still innumerable outstanding questions with direct importance to the treatment of patients with AML. Many of these challenges relate to how specific genetic lesions translate to clinical features and response to treatment, and despite large studies exploring the prognostic impact of mutations and groups of mutations, it is still not clear how this is related to disease biology. For example, it is unclear why patients with *NPM1* mutations are associated with such high rates of remission, and why *FLT3*-ITD mutations are associated with such high rates of disease recurrence. Nor is it clear why some combinations of mutations are more prevalent than others, although presumably lesions that are mutually exclusive have either functional overlap or are disadvantageous to the cell in combination.

1.8 The cell cycle

The decision of a cell to divide and the ability of a cell to divide are fundamental biological processes. The cell cycle also serves as an integration point that incorporates and translates multiple upstream signals, and it is highly regulated during the execution of division. The normal cell cycle may therefore go awry in the context of genetic lesions.

Precise duplication of DNA during each cell division is essential for genomic stability. This is achieved through the division of each cell cycle into discrete steps that are tightly regulated. During the synthesis or S phase, the chromosomal DNA of the cell is duplicated. Under normal circumstances, these duplicated chromosomes are divided equally between daughter cells in the mitotic or M phase. Prior to the S phase, there is a first gap phase (G_1), and between the S phase and the M phase there is a second gap phase (G_2). These gap phases are pauses that allow for checks of integrity and repair of genetic damage to occur prior to the next stage of the cell cycle. In addition, cells may exit the cell cycle and enter G_0 during quiescence, senescence, or differentiation (Figure 1.1).

1.8.1 The G_1-S phase checkpoint

The control of entry into the cell cycle is achieved through tightly regulated initiation events at multiple origins of replication throughout the genome. In late mitosis and early G_1, pre-replicative complexes (pre-RC) are assembled at origins of replication, which involves sequential binding of the origin of recognition complex (ORC, made up of proteins ORC1-6) and the proteins CDC6 and CDT1 (Blow & Dutta, 2005) (Figure 1.2). These then recruit and load MCM2-7, a heterohexameric complex of proteins with helicase activity, to origins of replication (Perkins & Diffley, 1998), creating the pre-RC. The chromatin at this ORC is now 'licensed' for DNA synthesis during S phase. Once MCM2-7 is loaded onto the DNA, CDC6 and CDT1 dissociate and are no longer required for MCM2-7 to remain bound to the DNA and for the origin to remain licensed (Blow & Dutta, 2005). On subsequent activation of MCM2-7 through phosphorylation by CDC7 kinase, a conformational change leads to the recruitment of DNA polymerase alpha and other members of the replication machinery to the complex and the initiation of DNA synthesis (Machida *et al*, 2005). This activation by CDC7 is believed to be non-redundant. After the initiation of replication, the MCM2-7 complex travels along the DNA in a 3' direction from the origin, with the consequence that the origins are left in an unlicensed state.

It is important to prevent reloading of MCM2-7 and re-replication of the same section of DNA in a single cell cycle. Thus the ORC, CDC6, and CDT1 are regulated in a cell cycle-dependent manner. One of the most important mechanisms to prevent MCM2-7 re-loading, and hence re-licensing, is the binding of CDT1 by the regulatory protein geminin (McGarry & Kirschner, 1998; Wohlschlegel *et al*, 2000). Geminin is itself subject to cell cycle-dependent regulation such that it is expressed at high levels and can only bind to CDT1 during S, G_2, and M phases (McGarry & Kirschner, 1998). Geminin is ubiquitinated at the metaphase to anaphase transition leading to its inactivation. Thus, in late M phase and early G_1, geminin is no longer available to bind CDT1, which is once again free to load MCM2-7 onto DNA and license origins at the beginning of the next cell cycle.

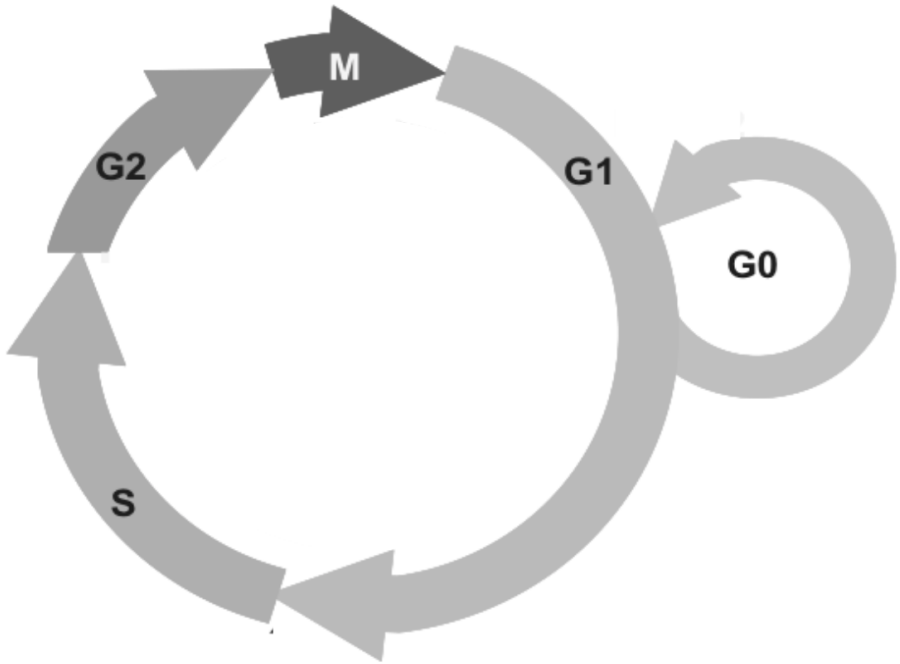


Figure 1.1 The Cell Cycle. The cell cycle can be divided into the synthesis (S) phase in which DNA is duplicated and the mitosis (M) phase in which cells divide into two daughter cells. These phases are separated by two gap phases (G_1 and G_2) in which growth and repair occur. G_0 is another gap phase denoting an out-of-cycle state, e.g. quiescence or senescence. (Figure adapted from Rodriguez-Acebes *et al*, 2010).

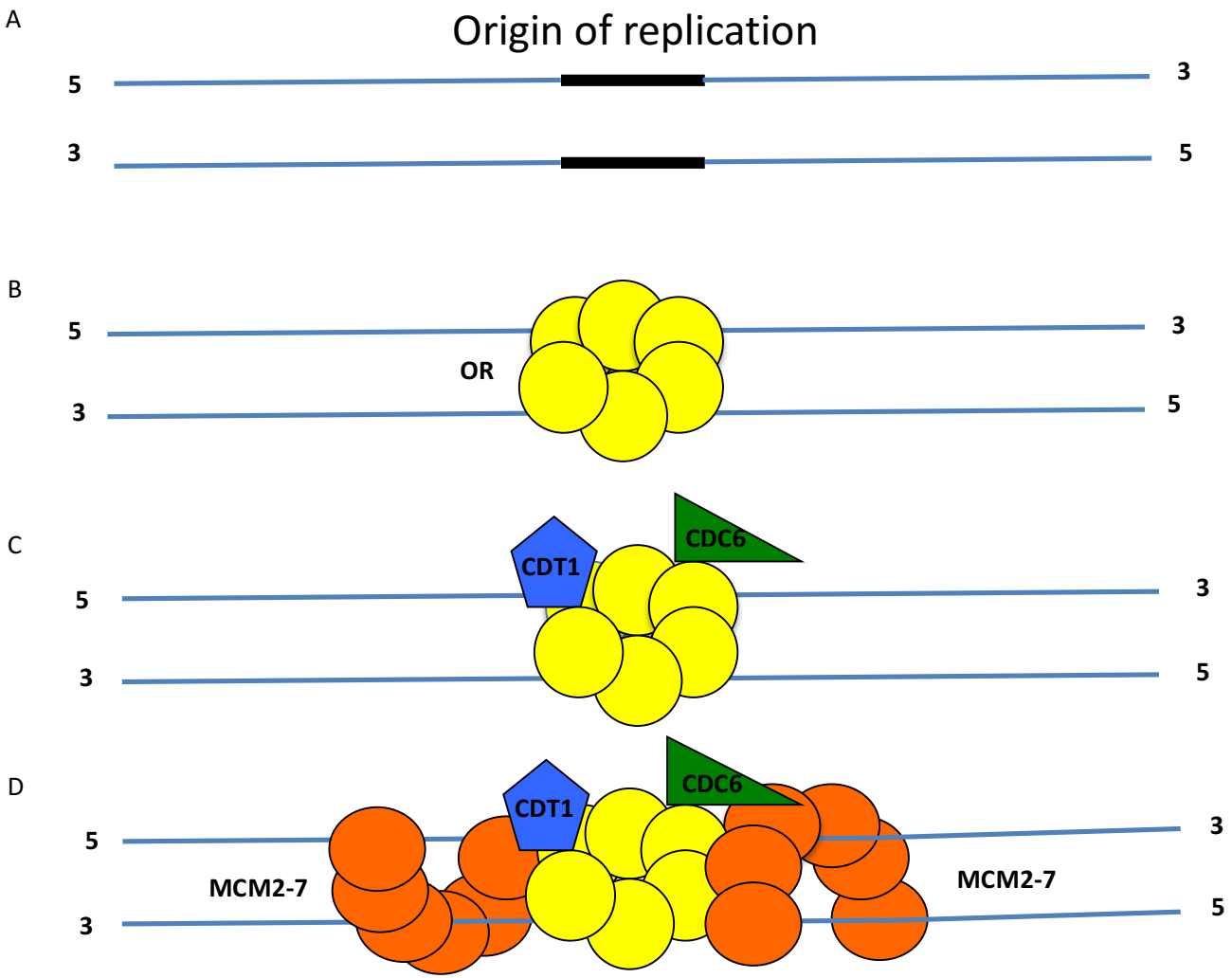


Figure 1.2 (continued on next page)

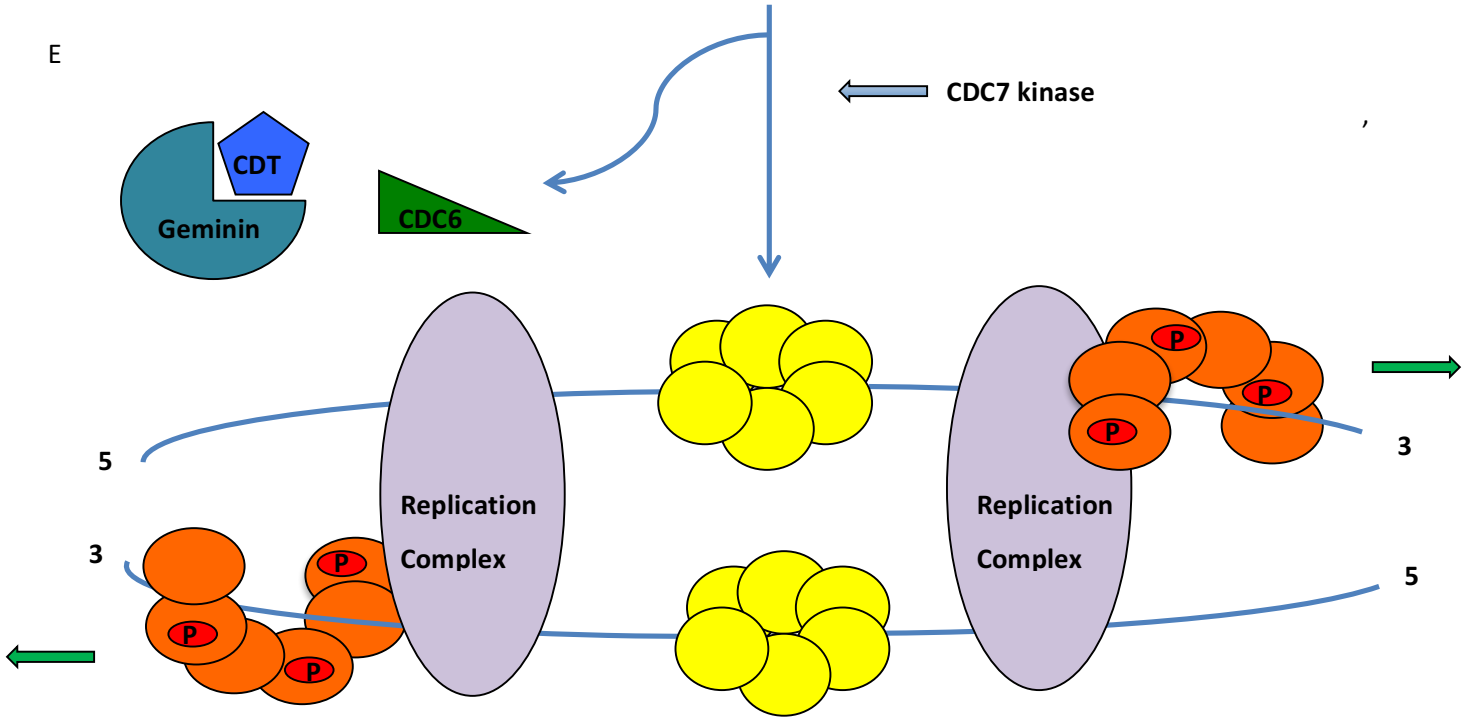


Figure 1.2 The G_1-S phase checkpoint. There are up to 100,000 origins of replication throughout the genome in each cell (A). The origin recognition complex (ORC) binds to an origin of replication (B) and recruits CDT1 and CDC6 (C). This complex then recruits MCM2-7 to the origin (D). After phosphorylation of MCM2, MCM4 and MCM6 by the kinase CDC7, CDC6 and CDT1 dissociate and new components are recruited to the DNA synthesis/sliding clamp complex (E). In addition to Polymerases α, δ and ε, the other components of the replication machinery are the GINS complex (consisting of the four proteins SLD5, PSF1, PSF2 and PSF3), MCM10, CDC/SLD2 and DPB11/SLD2. Once all components are recruited, the sliding clamp moves in a 3' direction replicating DNA (direction of travel indicated by green arrows).

1.8.2 The G_1-S phase checkpoint in disease

Downregulation of CDT1 at the G_1-S transition is a key event in preventing re-licensing of replicated origins. In addition to the binding of CDT1 by geminin, downregulation is also achieved by cell cycle-dependent proteolysis. The inhibition of either system of control appears sufficient to allow re-licensing and re-replication of DNA. Both insufficient licensing and re-replication may contribute to disease.

1.8.2.1 Insufficient origin licensing

As MCM2-7 proteins are essential for DNA replication, the prevention of their loading onto DNA completely prevents replication (Chong *et al*, 1995; Kubota *et al*, 1995; Madine *et al*, 1995; Ekholm-Reed *et al*, 2004a; Tsuji *et al*, 2006; Stuermer *et al*, 2007). However, a reduction rather than an absence of MCM2-7 loaded onto DNA may have unpredictable or detrimental consequences by reducing the number of replication origins available for use.

There is a considerable amount of redundancy in the system, both in terms of replication origins and also the number of MCM2-7 heterohexamers. For example, the replication forks initiated from a single origin of replication have the potential to replicate approximately 1.5 Mb of DNA, but in a mammalian cell, replication origins are spaced on average 30-150 kb apart. Furthermore, there are up to 20 times more MCM2-7 molecules loaded onto DNA in G_1 than there are active replication origins, and cells are able to synthesise DNA at normal rates when the level of MCM2-7 is reduced (Cortez *et al*, 2004; Tsao *et al*, 2004; Ge *et al*, 2007). This redundancy may allow cells to deal with problems arising during S phase. Replication forks that encounter DNA damage have the potential to stall irreversibly (Lambert & Carr, 2005). If such stalling happens in the context of two converging forks, replication of the intervening DNA is potentially difficult. As there is currently no known system that identifies the unreplicated portion of DNA, further licensing of DNA by loading more MCM2-7 at this stage could potentially lead to re-replication of DNA. The normal system of control therefore

dictates that all origins need to be licensed prior to entry into S phase, and as such it should not be possible to license a new origin between the two stalled forks.

However, inhibition of replication forks promotes the activation of dormant origins that do not fire in unperturbed phases (Anglana *et al*, 2003; Woodward *et al*, 2006; Ge *et al*, 2007; Gilbert, 2007; Ibarra *et al*, 2008). The activity of these dormant origins is dependent on the full complement of MCM2-7 being loaded onto DNA. In the absence of replicative stress, dormant origins are passively replicated by forks from neighbouring origins and therefore do not normally fire. Under conditions of replicative stress however, they become essential for complete replication and cell survival and, if MCM2-7 loading is reduced, fewer replication origins are used which results in DNA strand breaks, checkpoint activation, genetic instability, and cell death (Shreeram *et al*, 2002; Tanaka & Diffley, 2002; Feng *et al*, 2003; Teer *et al*, 2006; Bailis *et al*, 2008).

1.8.2.1.1 Animal models of insufficient origin licensing

Murine models have produced data that is consistent with these ideas. Mice heterozygous for an hypomorphic *Mcm4* allele produce a destabilised protein that leads to a reduction to approximately 50% of functional MCM2-7 heterohexamers being loaded onto DNA (Shima *et al*, 2007). A different mutant *Mcm4* has also been described that is able to generate a non-functional helicase whilst not affecting the stability of the mutant protein itself, thus locking other MCM proteins into a functionally dead complex equating to a functional loss of all members of the MCM2-7 complex (Bagley *et al*, 2012). A further model, this time assessing a hypomorphic MCM2 protein, exhibited similar defects in dormant origin usage (Pruitt *et al*, 2007; Kunnev *et al*, 2010). In all three models, there was evidence of increased DNA damage and genome instability. There was also some evidence of a stem cell deficiency, with an overall decrease in cellular proliferation and a possible depletion of the stem cell population. Perhaps unsurprisingly, mice in these models develop malignancies; 80% of female mice with hypomorphic MCM4 died of mammary adenocarcinoma (Shima *et al*, 2007), and MCM2 mutant mice developed T and B cell lymphomas (Pruitt *et al*, 2007).

1.8.2.1.2 Insufficient licensing in human disease

Mutations leading to a truncated form of MCM4 have also been shown to be responsible for human disease in which patients present with natural killer cell deficiency, adrenal insufficiency and growth retardation, in addition to genome instability (Casey *et al*, 2012; Gineau *et al*, 2012; Hughes *et al*, 2012). This truncated form of MCM4, although apparently incorporated into the helicase and loaded onto DNA, leads to increased levels of chromosome breakage and impaired cell cycle progression.

Other members of the pre-RC have also been implicated in human disease. Meier-Gorlin syndrome (MGS) is a rare disorder linked to mutations leading to defective non-MCM pre-RC proteins (ORC1, ORC4, ORC6, CDT1 and CDC6) and is characterised by primordial dwarfism, mild to severe microcephaly, and hypoplasia of the ear and patella (Bicknell *et al*, 2011a; Bicknell *et al*, 2011b; Guernsey *et al*, 2011). However, it is noteworthy that although some features of MCM-deficient mice are present in cells from affected individuals, including impaired licensing and altered S-phase progression, the patients do not display chromosomal instability or have a demonstrably increased risk of cancer. It is possible that the reduction in licensing seen in MGS is only critical to certain cells during development. It is also possible that the phenotype of MGS may reflect other functions of the pre-RC unrelated to their role in origin licensing. For example, ORC1 has a role in the regulation of centrosome duplication and the *ORC1* mutations found in MGS promote centrosome reduplication (Hemerly *et al*, 2009; Hossain & Stillman, 2012; Kuo *et al*, 2012). In addition, ORC6 has a role in cytokinesis (Prasanth *et al*, 2002), CDT1 is important for microtubule attachment to kinetochores (Varma *et al*, 2012), and CDC6 has a role in regulating the checkpoint kinase ATR (Clay-Farrace *et al*, 2003; Oehlmann *et al*, 2004). It is also noteworthy that all of the proteins mutated in MGS have been implicated in cilia formation that in turn are important for a number of signalling pathways, including Hedgehog signalling (Stiff *et al*, 2013).

1.8.2.2 Re-licensing in S and G_2 and re-replication

Proteolysis or inhibition of CDT1 is the predominant mechanism by which human cells downregulate the licensing system in S phase in order to prevent the potentially catastrophic re-licensing and re-replication that would otherwise occur. Proteolysis of CDT1 is coupled to DNA synthesis and cell cycle progression in at least two ways, both of which act by ubiquitination (Nishitani *et al*, 2006). CDT1 is a substrate of the CUL4-DDB1-CDT2 ubiquitin ligase and its activation requires that it is recruited to PCNA (proliferating cell nuclear antigen), a DNA polymerase factor, and thus links the degradation of CDT1 to ongoing DNA synthesis (Hu *et al*, 2004; Arias & Walter, 2006; Hu & Xiong, 2006; Senga *et al*, 2006). Another mechanism of ubiquitination requires the phosphorylation of CDT1 by CDK2-cyclin A kinase and its recognition by the SCF^{SKP2} complex. As CDK2-cyclin A kinase is active during S and G_2 phase, this system also couples the degradation of CDT1 to DNA replication and S-phase progression (Li *et al*, 2003; Liu *et al*, 2004; Sugimoto *et al*, 2004; Takeda *et al*, 2005). However, the main route to CDT1 proteolysis is likely to be the CUL4-DDB1-CDT2 ubiquitin ligase, as loss of any of these components leads to re-replication whereas the loss of SKP2 does not.

As described above, geminin is active during the S-G_2-M phases and binds to CDT1, thus preventing the loading of MCM2-7 onto DNA during these phases (McGarry & Kirschner, 1998; Wohlschlegel *et al*, 2000; Tada *et al*, 2001). The loss of geminin results in re-licensing and re-replication (Melixetian *et al*, 2004; Zhu *et al*, 2004). The activity of geminin is halted at the metaphase to anaphase transition following ubiquitination by APC/C, thus freeing CDT1 to license DNA in preparation for the next round of DNA replication. APC/C is in turn regulated by EMI1 (Machida & Dutta, 2007). The main role of EMI1 appears to be the inhibition of APC/C in G_1 in order that there is sufficient CDK activity to allow entry into S phase. However, loss of EMI1 leads to a failure to regulate APC/C, which destabilises geminin during S phase and promotes re-replication.

Although the deregulation of CDC6 plays a minor role in preventing re-licensing, it may allow limited re-replication. Part of the control mechanism appears to be through the export of CDC6 from the nucleus during S-G_2 phase, and overexpression of CDC6,

which results in higher nuclear levels during these stages of the cell cycle, increases the severity of re-replication seen following dysregulation of CDT1 (Saha *et al*, 1998; Petersen *et al*, 1999). More recently, an alternative mode of regulation by SCF-dependent degradation of CDC6 has been shown to prevent re-replication (Walter *et al*, 2016).

CDC6 overexpression also causes heterochromatinisation and repression of the INK4A/ARF tumour suppressor locus (CDKN2A) (Gonzalez *et al*, 2006b). In addition, CDC6 has been shown to be involved in the activation of checkpoint kinases in response to the inhibition of replication (Clay-Farrace *et al*, 2003; Oehlmann *et al*, 2004). Finally, CDC6 may stimulate inappropriate recovery from cell cycle arrest mediated by p21 in response to DNA damage by releasing p21 from CDK (Kan *et al*, 2008). All of these effects could lead to genetic instability. There is also recent evidence that CDC6 regulates the initiation of ribosomal DNA transcription providing a link beyond DNA replication to protein expression (Huang *et al*, 2016).

1.8.2.2.1 Animal models of re-licensing in S and G2 and re-replication

Overexpression of CDT1 in a mouse model results in tumour formation (Arentson *et al*, 2002). In addition, mice that specifically overexpress CDT1 in T-cells develop thymic lymphoblastic lymphoma in a *p53*-null background (Seo *et al*, 2005). Crucially however, it is not known whether replication licensing is the key rate-limiting step for progression through G_1 and hence whether increasing the rate of loading of MCM2-7 onto DNA may significantly alter the rate of cell division. It may be therefore, that the apparent malignant potential of CDT1 is a consequence of the genetic instability it induces. Indeed, malignant cells overexpressing CDT1 in this same mouse model displayed severe chromosomal aberrations and genetic instability.

The downregulation of geminin following the transduction of *Hoxb4* into murine foetal liver cells resulted in increased proliferation potential in murine haematopoietic stem and progenitor cells, which was postulated to be through the increased loading of pre-RC onto DNA (Ohno *et al*, 2010). *Gmnn* null mouse embryos stop dividing at the early

blastula stage with greater DNA content than normal, premature differentiation as trophoblast cells, and no markers of embryonic stem cells (Gonzalez *et al*, 2006a; Hara *et al*, 2006). In a subsequent inducible model, deletion of geminin from haematopoietic cells using a conditional floxed geminin allele, with loxP sites flanking exons 5, 6, and 7 (encoding the binding site for CDT1) and a *Cre* recombinase under an Mx1 promoter led to anaemia and thrombocytosis in mice (Shinnick *et al*, 2010). Intriguingly, although there was some evidence of over-replication of DNA in methylcellulose colonies of CD34+ cells from these mice, and there was a dramatic decrease in colony forming ability including a complete absence of erythroid colonies, the stem and progenitor cell compartment in the mice was preserved. The geminin null cells were able to successfully reconstitute haematopoiesis in lethally irradiated mice (although there was an engraftment defect on secondary transplantation), and the megakaryocytes and erythroblasts had normal DNA content. In *ex vivo* culture there was, however, clear evidence of overreplication of DNA in isolated CD34+ cells, with a significant population of cells with DNA content greater than 4n.

In addition to its role in cell cycle regulation, models have also revealed interactions of geminin with other protein targets including other homeobox members, members of the polycomb group 1 complex, and members of the SWI/SNF chromatin remodelling complex (Seo & Kroll, 2006). Through these interactions, geminin has been shown to influence transcriptional programmes and have a key role in neural development, organogenesis, and axis patterning (Kushwaha *et al*, 2016). It has no clear role in haematopoietic transcriptional programmes, although there is some evidence from *in vitro* siRNA experiments in mouse bone marrow that HOXA9 may induce the activity of haematopoietic progenitors in part through down regulation of geminin (Ohno *et al*, 2013).

1.8.2.2.2 Mutations in the replication licensing system in cancer

A review of the COSMIC (catalogue of somatic mutations in cancer) database (http://cancer.sanger.ac.uk/cosmic) shows that, with the exception of *APC*, mutations in the genes encoding the pre-RC and the main proteins controlling the pre-RC are

infrequent in human cancer (Table 1.10), particularly when compared with the mutation rates of some of the more frequently mutated genes found in various cancer types including *TP53*, *KRAS* and *BRAF* (Alexandrov *et al*, 2013). However, the apparently high levels of non-redundancy in the system, particularly with the central role of CDT1 for preventing re-replication, may mean that mutations that result in, for example, loss of functional geminin or failure of CDT1 to be lysed or bound by geminin, may lead to lethal levels of re-replication and therefore cells acquiring such a mutation do not survive sufficiently long to gain a potential competitive advantage from genomic instability.

However, there is some evidence that dysregulation of this crucial pathway does exist in cancer, and it is unclear whether this dysregulation is a consequence or a cause of the oncogenic process, or indeed both. For example, it is possible that malignant cells lose the ability to differentiate and therefore continue to divide and express MCM proteins. In this scenario, the MCM proteins are not directly oncogenic but simply a marker of active proliferation. An alternative explanation is that the deregulation of replication licensing actively promotes the development of cancer by inducing re-replication and chromosomal instability.

Of note, some of the genes that are frequently mutated in cancer have been shown to be involved in abnormalities of G_1-S phase control and DNA replication, indicating that there are likely to be other routes to dysregulation of cell cycle progression through G_1-S phase. For example, overexpression of cyclin D1, an oncogene that is frequently mutated in cancer, can result in origin re-licensing and re-replication (Aggarwal *et al*, 2007), presumably at levels that are sufficient to result in genetic instability without resulting in cell death. Again, the crux of the mechanism appears to involve CDT1 stabilisation in S phase as a result of the loss of CUL4 expression.

Cyclins may also be linked to insufficient licensing. For example, the overexpression of cyclin E is associated with a reduction in the loading of MCM2-7 onto DNA during late M and G_1 phases of the cell cycle (Ekholm-Reed *et al*, 2004a). Consistent with this reduction is a decreased rate of S phase progression, as well as genetic instability, and accelerated tumour formation in mouse models of cyclin E abnormalities (Spruck *et al*, 1999; Ekholm-Reed *et al*, 2004b; Loeb *et al*, 2005).

Table 1.10. The frequency of mutations in replication licensing proteins and selected frequently mutated genes

	Gene	Number of samples assessed	Number of coding mutations detected	Coding mutations (%)
Replication licensing proteins	ORC1	35730	151	0.42%
	ORC2	35732	108	0.30%
	ORC3	35731	117	0.33%
	ORC4	35731	72	0.20%
	ORC5	35731	85	0.24%
	ORC6	35731	35	0.10%
	MCM2	36824	6	0.02%
	MCM3	35752	129	0.36%
	MCM4	35824	174	0.49%
	MCM5	35753	119	0.33%
	MCM6	35752	126	0.35%
	MCM7	35752	118	0.33%
	CDT1	35752	72	0.20%
	CDC6	36220	84	0.23%
	GMNN	35752	36	0.10%
	CDC7	36633	122	0.33%
	EMI1	36814	67	0.19%
	APC	54158	4700	11.52%
Selected frequently mutated genes	TP53	121490	30125	24.80%
	KRAS	204218	38301	18.75%
	NRAS	102740	4888	4.76%
	BRAF	238724	43242	18.11%
	PTEN	66367	3583	5.40%
	CDKN2A	69867	5027	7.20%

Data extracted from the COSMIC (catalogue of somatic mutations in cancer) database October 2016 (http://cancer.sanger.ac.uk/cosmic)

1.9 Cell cycle markers in oncology

The well-defined biology of the G_1-S phase transition means that analysis of the expression of relevant proteins may enable the definition of the stage of the cell cycle of a particular cell. Many of these cell cycle proteins can now be detected by immunohistochemistry of fixed tissue sections (Williams & Stoeber, 2007; Rodriguez-Acebes *et al*, 2010), allowing for direct analysis as well as assessment of archived samples. MIB-1, the antibody that recognises the MKI67 protein (a protein associated with ribososmal RNA transcription and the perichromosomal layer and that is nuclear only in proliferating cells (Booth *et al*, 2014)) is the gold standard immunohistochemical marker for proliferation used in routine clinical practice. It identifies actively proliferating cells as opposed to cells that are in G_1 and licensed for replication only and, in addition, may not be positive at the very earliest stages of G_1 (Figure 1.3). However, from the known expression of other proteins, as outlined in Figure 1.2, additional markers can be added in an attempt to better define the stages of the cell cycle. For example, antibodies to MCM2 label cells that are actively in cycle (G_1-S- G_2-M phases). Expression of MCM2 is lost when cells enter a quiescent non-proliferating state (G_0) and this is readily demonstrable on fixed tissue sections (Figure 1.4). Although there is a correlation between MCM2 and MIB-1 expression in tissue sections, they are not surrogates for one another. For example, in some tumour types MCM2 may be expressed in the majority of cells, whilst MIB-1 is expressed by <10% of cells in the same tissue section (Dudderidge *et al*, 2005a). The presence of a weaker correlation suggests a tumour phenotype with a low proliferation rate and/or cells arrested in early G_1. The origin licensing inhibitor geminin is only detectable once cells have passed the G_1-S phase checkpoint into S/G_2/M phases (Figure 1.3), thus preventing re-replication.

These three markers, MCM2, MIB-1, and geminin, may thus be used on archived histological sections to define populations of cells at various stages of the cell cycle (Table 1.11). They may also be used to derive features of cell cycle kinetics such as the number of cells out of cycle (MCM2 negative), cells in cycle without actively cycling (MCM2 positive, MIB-1 negative), and the number of cells that have progressed through G_1 into the S-G_2-M phases (MCM2 positive, MIB-1 positive, and geminin positive) (Table 1.12).

Table 1.11 Cell cycle markers that define cells at different stages of the cell cycle

	Antibody	**Cells stained**
Biomarker	MCM2	Cells with origin of replication licensed for DNA synthesis and cell division Positive in G_1/S/G_2/M, not in G_0
	MIB-1 (MKI67)	Actively proliferating cells Positive in G_1/S/G_2/M, not in G_0
	Geminin	Cycling cells that have progressed beyond G_1 Positive in S/G_2/M, not in G_1 or G_0

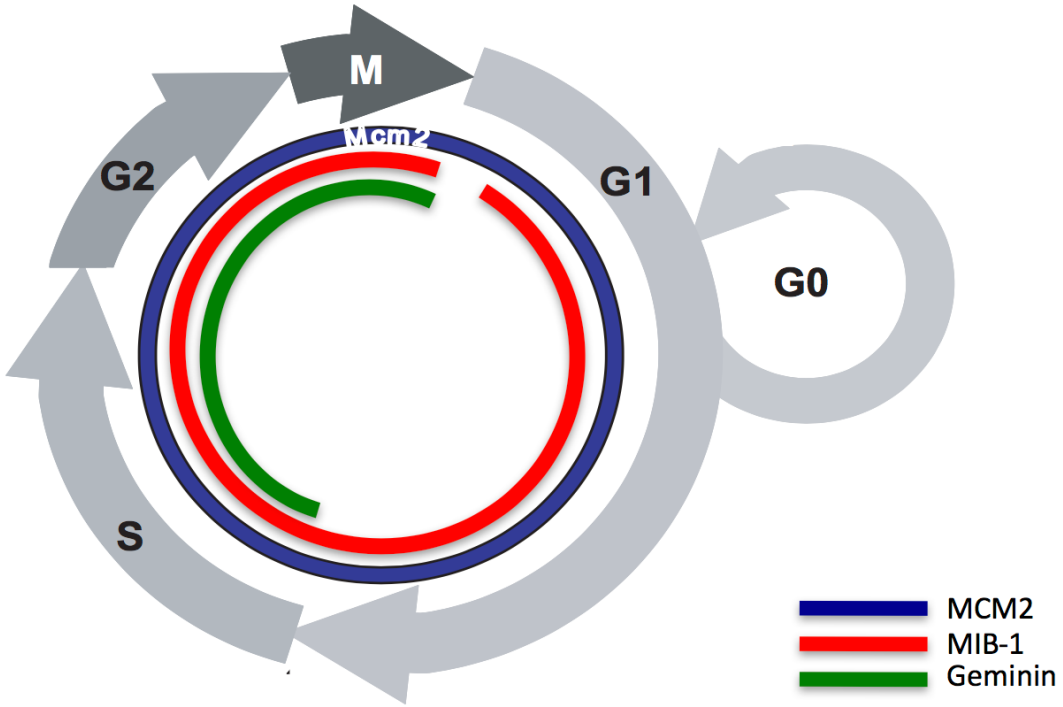


Figure 1.3 Expression of cell cycle markers in relation to the phases of the cell cycle. Mcm2 is expressed in all cells in all of the cell cycle phases except G_0, MIB-1 detects the protein MKI67 and is expressed in all actively proliferating cells but not those cells in the earliest stages of G_1 or arrested in G_1. Geminin is expressed only in cells that have progressed beyond G_1. (Figure adapted from Rodriguez-Acebes *et al*, 2010).

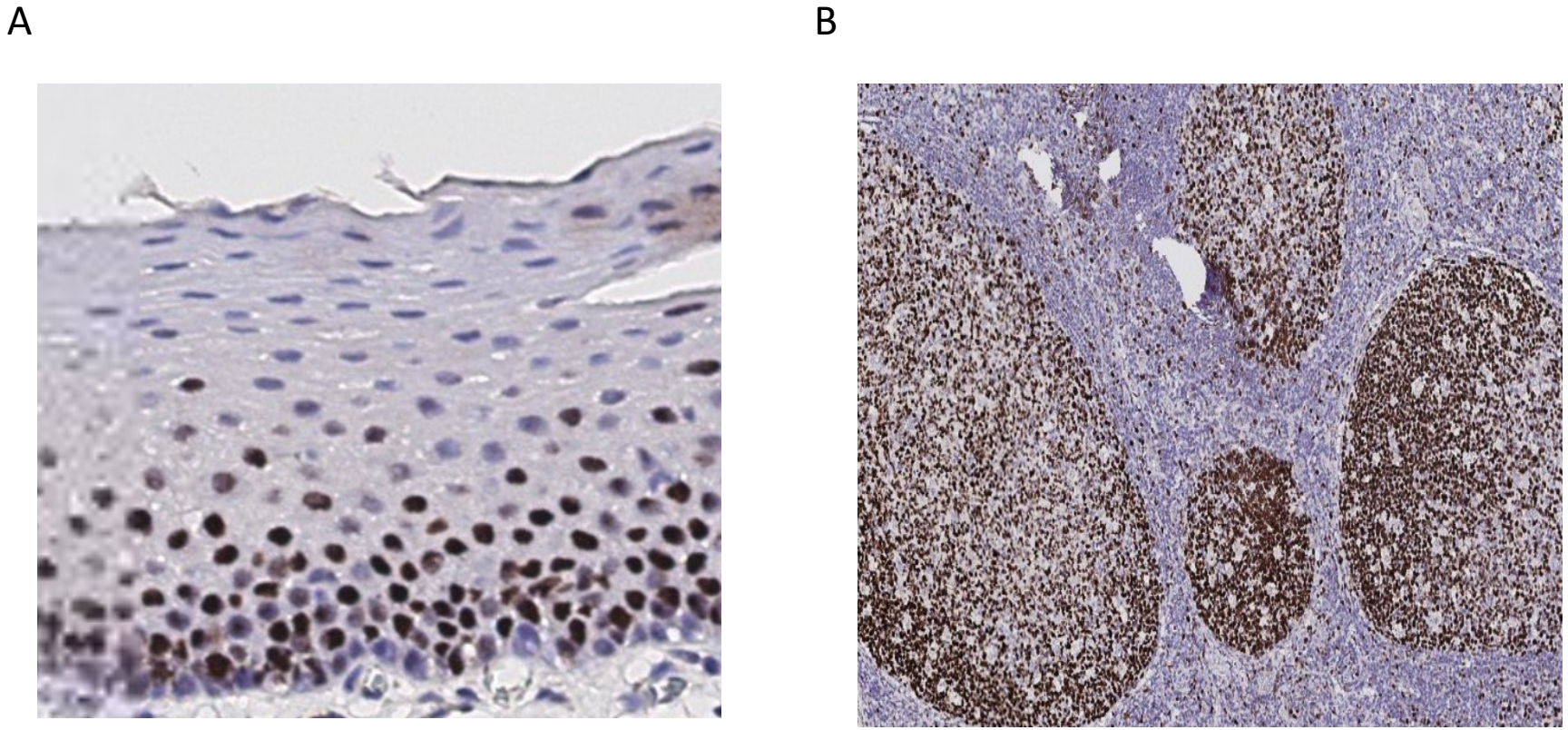


Figure 1.4. Expression of MCM2 in normal epithelial and glandular tissue. MCM2 expression using immunohistochemistry in normal human tonsil showing the expression (darkly stained cells) in (A) epithelium (x40) and (B) glandular tissue (x20). In epithelium it is expressed in the basal proliferating layer but lost as cells differentiate (enter G_0) and migrate towards the epithelial surface. Expression in glandular tissue is generally restricted to the follicles, the region in which cells are proliferating.

Table 1.12 Derived values from cell cycle marker LIs that define cell cycle kinetics

Relationship of biomarker	Significance of relationship
MIB-1 LI/MCM2 LI	The proportion of non-quiescent cells that are actively proliferating
Geminin LI/MIB-1 LI	Indicates the relative length of G_1 phase as a surrogate for speed of proliferation
MIB-1 LI – Geminin LI	Represents the proportion of cells that are transiting G_1

1.9.1 Expression of licensing proteins in cancer

Many studies have shown that there is an inappropriate expression of MCM2-7 and other pre-RC proteins in a wide variety of premalignant dysplasias and cancers, and these have been used to try and improve both diagnostic tests and predicted response to treatment. For example, it has been shown that the detection of an elevated level of MCM5 in urine sediments is predictive of bladder cancer (Stoeber *et al*, 1999; Stoeber *et al*, 2002), and this principle can be extended to gastric aspirates, biliary secretions, and stool samples (Davies *et al*, 2002; Williams *et al*, 2004; Ayaru *et al*, 2008). Such markers can also be combined with morphology to improve the accuracy of screening tests of, for example, cervical smears (Williams *et al*, 1998).

There have been numerous studies associating the aberrant expression of proteins linked to G_1-S phase with the aggressiveness of a malignancy. For example, expression levels of MCM2 and geminin predict for adverse prognosis in breast cancer (Gonzalez *et al*, 2003; Gonzalez *et al*, 2004), as does CDC6 in ovarian cancer (Deng *et al*, 2016). Although normally predicting for poorer outcome, this is not the case in all tumour types studied. For example, higher expression of geminin correlates with improved survival in high-grade astrocytomas (Shrestha *et al*, 2007).

This principle has been extended to study the association between combinations of markers of cell cycle status/progression and clinical outcome. The larger studies containing >100 patients are summarised in Table 1.13. The biological features seen and the prognostic impact are partially dependent on the exact markers studied, the cut-off for significance, the tumour type studied, and the type of analysis done including, for example, whether multivariate analysis was performed. In one study of penile cancer, the markers MCM2, geminin and Ki-67 (MIB-1) were associated with features of aggressive disease (Kayes *et al*, 2009), a finding confirmed by others (May *et al*, 2013). However, the results of two studies assessing the impact of cell cycle status in NSCLC, which included MCM2, were contradictory. The larger of the two studies concluded that patients with lower levels of MCM2 had longer median survival (Ramnath *et al*, 2001). In contrast, a later study using the same cut-off for high MCM2 (25%) that had fewer patients but examined a greater number of other cell cycle biomarkers found a non-significant effect of MCM2 on survival except when considered in conjunction with the marker gelsolin, an actin binding protein that regulates actin filament assembly and disassembly (Yang *et al*, 2006). Such differences may relate to the smaller numbers in the study, positive or negative associations between markers, or the need to combine markers to gain more complete information of cell cycle dynamics in tumour samples. In general, the larger studies using combined cell cycle markers have shown a correlation of the expression of DNA replication licensing proteins with more aggressive disease that in some cases is independent of other known prognostic factors.

1.9.2 Expression of DNA replication licensing proteins in haematological malignancies

In a study of various B-cell malignancies, the patterns of expression of cell cycle licensing proteins were associated with disease phenotype. In samples from patients with CLL, most cells were arrested in G_1 (Obermann *et al*, 2005a). In contrast, in samples from patients with DLBCL or Burkitt's lymphoma, the majority of cells were actively cycling as defined by a higher geminin to Ki-67 (MIB-1) ratio. Crucially,

Table 1.13 Key findings from larger studies (>100 patients) of cell cycle analysis using immunohistochemistry in various tumour types

Tumour Type	Number of Patients	Markers studied	Outcome	Reference
NSCLC	221	MCM2 Ki-67(MIB-1)	MCM2 was an independent prognostic marker for survival. Patients with LI<25% had longer median survival	(Ramnath *et al*, 2001)
Renal	176	MCM2 Ki-67 (MIB-1) Geminin	Increased expression of all markers associated with DFS. Only Ki-67 LI>12% was independent of clinical variables such as grade and capsular invasion	(Dudderidge *et al*, 2005b)
Lung adenocarcinoma	145	MCM2 Ki-67 (MIB-1)	High MCM2 and Ki-67 LIs were independent prognostic factors for poor outcome	(Hashimoto *et al*, 2004)
GIST	277	MCM2 Ki-67 (MIB-1) p16 (INK4A)	Increased LIs for MCM2 and Ki-67 associated with decreased DFS. Ki-67 but not MCM2 was independent on multivariate analysis	(Huang *et al*, 2006)
NSCLC	128	MCM2 Ki-67 (MIB-1)	Increased levels of MCM2 associated with non-significant inferior survival. No impact of Ki-67 on outcome	(Yang *et al*, 2006)
Ovarian Epithelial	143	MCM2 Ki-67 (MIB-1) Geminin	Markers were associated with tumour stage and ploidy	(Kulkarni *et al*, 2007)
Penile	141	MCM2 Ki-67 (MIB-1) Geminin	Markers associated with arrested tumour differentiation and aneuploidy Accelerated cell cycle progression was linked to increasing tumour size, stage, and depth of invasion	(Kayes *et al*, 2009)
Breast	182	MCM2 Ki-67 (MIB-1) Geminin	Higher rates of relapse in patients with both high MCM2 (>30%) and high geminin (>7%)	(Loddo *et al*, 2009)
Gastric	128	MCM2 Ki-67 (MIB-1) Geminin	Higher geminin LI (>25%) associated with worse prognosis	(Shomori *et al*, 2010)
Salivary	170	Ki-67 (MIB-1) Geminin	Geminin associated with tumour invasion and metastasis Higher geminin associated with decreased OS and independently predictive of survival and relapse	(Yamazaki *et al*, 2010)
Penile	158	MCM2 Ki-67 (MIB-1) Geminin	Markers associated with more aggressive disease but were not independent factors	(May *et al*, 2013)

DFS, disease-free survival; GIST, gastrointestinal stromal tumour; NSCLC, non-small cell lung cancer

these phenotypes were also associated with the response and curability of these diseases following treatment by standard chemotherapy.

In myeloid malignancies, MCM2 expression has been assessed in MDS, and MCM2 expression was associated with bone marrow cell apoptosis and peripheral blood cytopaenias (Suzuki *et al*, 2012). However, there have been no studies assessing the patterns of expression of cell cycle licensing proteins to analyse the cell cycle status of samples from patients with either MDS or AML.

1.10 The Cell Cycle in AML

In the normal haematopoietic system, proliferation is tightly linked to differentiation. In an asymmetrical cell division, one daughter cell retains stem cell properties while the other undergoes differentiation. It is also possible to have symmetrical division where both daughter cells are stem/progenitor cells or both differentiate, with the choices made being influenced by cytokines and the subset of stem cells (Rieger *et al*, 2009; Ito *et al*, 2016). In the context of AML, the balance between differentiation and cell cycle progression is grossly abnormal, with a drive away from differentiation towards proliferation.

There are also numerous interdependent mechanisms of cell cycle dysregulation in AML. These include abnormal signalling drives to enter or progress through the cell cycle, as well as impairment of crucial inhibitors of cell cycle progression (Table 1.14). Many of these have been targets for drug development, and some of the drugs currently in the most advanced phases of clinical development are outlined in Table 1.15.

Table 1.14 Examples of key mechanisms implicated in cell cycle dysregulation in AML

General mechanism of dysregulation	Affected gene	Normal protein function	Defect in AML	Implication for cell cycle	Reference
Signal Transduction	FLT3	Expansion and survival of early haematopoietic progenitor cells	Activating mutations Overexpression	Possible drive to excess proliferation	(Kelly & Gilliland, 2002)
	c-KIT	The tyrosine kinase receptor for stem cell factor. Expansion and survival of early haematopoietic progenitor cells	Activating mutations		
	RAS	Activates downstream effector pathways including PI3K and MAPK	Activating mutations in both N-RAS and K-RAS		
Cyclins	Cyclin A	Activation of Cdk2 in the regulation of S phase progression	Upregulated/overexpressed	Increased cell cycle progression	(Yang *et al*, 1999)
	Cyclin B	Activation of Cdk1 at the G_2-M phase transition	Upregulated/overexpressed		(Nakamura *et al*, 2010)
	Cyclin D	Activation of Cdk4/6 that is required for cell cycle progression	Upregulated/overexpressed		(Argiropoulos *et al*, 2010)
	Cyclin E	Activation of Cdk2 in the regulation of the G_1-S phase transition	Upregulated/overexpressed		(Iida *et al*, 1997)
Cyclin-dependent kinase (Cdk) inhibitors	p15	Inhibitor of Cdk4	Downregulated	Increased rate of progression into S phase	(Paul *et al*, 2010)
	p16	Inhibitor of Cdk4	Downregulated		

	p21 p27	Inhibitor of Cdk2 and Cdk4 Inhibitor of Cdk2 and Cdk4	Downregulated Downregulated		(Taniguchi *et al*, 1999) (Peterson *et al*, 2007) (Yokozawa *et al*, 2000)
Mitotic kinase	Aurora A Aurora B PLK1	Involved in function of the mitotic spindle including chromosome alignment and cytokinesis Chromosome attachment to microtubules Regulation of numerous facets of mitosis including mitotic entry and chromosome separation	Overexpressed Overexpressed Overexpressed	Mitotic checkpoints over-ridden Chromosomal missegregation Aberrant entry into mitosis. Potential contribution to aneuploidy	(Yang *et al*, 2013) (Yang *et al*, 2007) (Renner *et al*, 2009)
DNA damage response	TP53	Inhibition of cell cycle progression in the event of DNA damage	Mutated and/or deleted	Reduced checkpoint activity and inappropriate cell cycle progression in the context of DNA damage	(Zhang *et al*, 2016)

Table 1.14 continued

Table 1.15 Examples of novel drugs targeting cell cycle proteins currently in trials of AML patients

Protein(s) targetted	Normal Function	Abnormality in AML	Drug	Mechanism of action	Trial Phase	Trial
Mdm2	E3 ubiquitin ligase that negatively regulates p53	Overexpressed in some subsets of AML	Idasanutlin	Small molecule antagonist of Mdm2	Phase 1	NCT01773408
Cyclin-dependent kinases	Serine/threonine kinases that are activated by Cyclins, e.g. Cdk4/6 activated by Cyclin D, and Cdk2 activated by Cyclin E	Downstream targets of activating kinase mutations and deregulated signalling cascades	Alvocidib	Flavinoid alkaloid inhibitor of Cdk9 and Cdk4/6	Phase 2	NCT01413880
Plk1 (polo-like kinase 1)	Regulator of mitotic entry, centrosome kinetics, and cytokinesis	Overexpressed in some subsets of AML	Volasertib	Small molecule dihydropteridinone derivative inhibitor of Plk1	Phase 3	NCT01721876
Aurora kinases	Aurora kinase A required for proper function of mitotic spindle, chromosome alignment, and cytokinesis Aurora kinase B required for chromosome attachment to microtubules	Both are overexpressed in AML	Alisertib Barasetib	Selective inhibitor of Aurora kinase A Selective inhibitor of Aurora kinase B	Phase 1 Phase 2/3	NCT01779843 NCT00952588

1.10.1 The rationale for studying DNA replication licensing proteins in AML

The cell decision to traverse from G_1 to S phase lies at the crossroads of proliferation and differentiation. The cell cycle machinery acts as an integration pathway for the information transduced through upstream signalling pathways that determines whether a cell undergoes cell division or differentiates (Williams & Stoeber, 2007). In addition to the wide array of cell cycle abnormalities outlined above, the effects on this point of integration may reflect the combined influence of cytogenetic and molecular lesions found within a cell. Thus, analysing the cell cycle licensing proteins in the context of well-defined cytogenetic and molecular lesions may inform our understanding of disease biology. Defining the cell cycle according to cell cycle licensing proteins may also inform on the response to therapy. The intensive induction chemotherapy regimens described earlier are to some extent dependent on cells being in a state of actively cycling in order to be effective. This can be appreciated by considering the mechanism of the two main drugs used for induction chemotherapy in AML: daunorubicin and Ara-C.

Daunorubicin is an anthracycline, a class of drugs that act by inhibiting topoisomerase II. The action of this enzyme under normal physiological conditions is to relax supercoils in DNA, thus allowing for transcription or replication to occur. The drug stabilises the topoisomerase complex, preventing the double helix resealing correctly and restricting replication. In addition, anthracyclines bind to and intercalate DNA, and on intercalation may also displace histones from chromatin (Pang *et al*, 2013).

Ara-C is converted to cytosine arabinoside triphosphate by the enzyme deoxycytidine kinase. The triphosphate is then competitively incorporated into the DNA strand during chain elongation, which results in chain termination and a block in DNA synthesis (Kufe *et al*, 1980; Major *et al*, 1981; Major *et al*, 1982). Sustained high cellular concentrations of Ara-C triphosphate relative to the deoxycytidine form are thought to favour drug incorporation into replicating DNA, thereby initiating the leukemic cell death associated with therapeutic response (Gunji *et al*, 1991). A further method of Ara-C action is by inhibition of DNA polymerase, in particular polymerase α (Yoshida *et al*, 1977; Allaudeen *et al*, 1982). The marked differences in response to these two drugs according to clinical and biological features may reflect resistance to apoptosis, for

example through inadequate TP53 response to DNA damage, intrinsic mechanisms of drug resistance such as MDR-1 expression or, in the case of Ara-C, possibilities also include lower levels of deoxycytidine kinase leading to decreased levels of the active triphosphate form, higher levels of cytidine deaminase that may inactivate Ara-C, or higher levels of deoxycytidine triphosphate that results in more resistance to competition from the Ara-C triphosphate form.

However, as with the response described above for patients with lymphoproliferative disorders, the differences may also reflect varying vulnerabilities based upon the relative amount of time spent in a particular phase of the cell cycle compared to normal cells, whether because of the rapidity of cell cycling and the resulting levels of drug incorporation, or due to spending more time in a phase of the cell cycle where the cell is more vulnerable to damage. Numerous studies have sought to relate the cell cycle status in patients with AML with response to treatment and clinical outcome, and these will be presented in detail in chapter 3. However, the results have been inconsistent and, for some features, directly contradictory. Furthermore, none have sought to define cell cycle status according to well-defined cell cycle licensing markers, nor have they been performed with knowledge of cytogenetic data and the major molecular prognostic markers *FLT3*-ITD and *NPM1*.

1.11 Summary and aims of thesis

The aim of the studies presented in this thesis was therefore to explore the relationship between the cell cycle dynamics of leukaemic blast cells from patients with AML, in terms of the expression of DNA replication licensing proteins, with clinical, cytogenetic and molecular features of the patients' disease, and further to assess this information in relation to response to induction therapy and overall clinical outcome.

Chapter 3 examines the current body of knowledge of clinical studies of the cell cycle in AML, and discusses possible reasons for inconsistencies between the results and how the previous methods used are open to inaccuracies. Results are then presented on studies assessing expression of cell cycle licensing proteins for defining the cell cycle

state in patients with AML. Chapter 4 describes a cohort of patients with a new diagnosis of AML treated at UCLH and known to have pre-treatment biopsies available for further analysis, and defines this population in terms of clinical and molecular features, response to treatment and outcome. Chapter 5 then presents the results of studies determining the expression of cell cycle licensing proteins in these patients and their correlation with the clinical features, major molecular mutations, and clinical outcome. Areas of further study suggested by the work in this thesis are then explored in chapter 6.

CHAPTER 2. MATERIALS AND METHODS

Specific materials and methods are described in the relevant results chapters.

2.1 Immunohistochemistry and immunocytochemistry

2.1.1. Reagents

Agarose (Bioline, London, UK)
Ammonium chloride (VWR International Ltd., Lutterworth, UK).
Bond™ Polymer Refine Detection kit (Leica Biosystems, Newcastle, UK).
Bond™ Primary Antibody Diluent (Leica Biosystems, Newcastle, UK).
Dulbecco's Phosphate Buffered Solution (PBS) without calcium/magnesium (PAA Laboratories Ltd., Somerset, UK)
EDTA (ethylenediaminetetraacetic acid, disodium salt) (Sigma Aldrich, Poole, UK)
Ethanol 100% (VWR International Ltd., Lutterworth, UK)
Formalin (37-40%) (VWR International Ltd., Lutterworth, UK)
Sodium phosphate (monobasic) (VWR International Ltd., Lutterworth, UK)
Sodium phosphate (dibasic/anhydrous) (VWR International Ltd., Lutterworth, UK)
Trypan Blue (0.4%) (Sigma Aldrich, Poole, UK)
Xylene (VWR International Ltd., Lutterworth, UK)

2.1.2 Antibodies

Cleaved Caspase-3 (Asp175) (Cell Signalling Technology, Hitchin, UK)
Geminin (Leica Biosystems, Newcastle, UK).
MCM2 (BD Biosciences, Oxford, UK)
MIB-1 (Dako UK Ltd., Ely, UK)

2.1.3 Buffers

Ammonium chloride red cell lysis buffer (10x): 8.02g ammonium chloride, 0.84g sodium bicarbonate, 0.37g EDTA (disodium) in 100ml DDW. Stored at 4°C.

Neutral buffered formalin (10%): 100ml of formalin (37-40% stock solution) in 900ml DDW, 4g sodium phosphate (monobasic), 6.5g sodium phosphate (dibasic/anhydrous). Stored at room temperature.

2.2 Molecular biology

2.2.1 Reagents

Agarose (Bioline, London, UK)
Bioline buffer and magnesium chloride (Bioline, London, UK)
BIOTAQ^{TM} polymerase (Bioline, London, UK)
Boric acid (VWR International Ltd., Lutterworth, UK)
Bromophenol blue (VWR International Ltd., Lutterworth, UK)
Chloroform (VWR International Ltd.., Lutterworth, UK)
DNA size standard kit – 400bp or 600bp (Beckman Coulter UK Ltd., Buckinghamshire, UK)
Diethylpyrocarbonate (DEPC) treated water (Invitrogen, Paisley, UK)
dNTPs (Bioline, London, UK)
DTAB (dodecyl-trimethyl ammonium bromide) (Sigma Aldrich, Poole, UK)
EDTA (ethylenediaminetetraacetic acid, disodium salt) (Sigma Aldrich, Poole, UK)
Glycerol (VWR International Ltd., Lutterworth, UK)
Ethanol 100% (VWR International Ltd., Lutterworth, UK)
Ethidium bromide (Invitrogen, Paisley, UK)
Hyperladder IV (Bioline, London, UK)
Primers (IDT, Leuven, Belgium)
Purelink^{TM} RNA Micro Kit (Life Technologies Ltd., Paisley, UK)
Purelink^{TM} DNase/Carrier RNA (Life Technologies Ltd., Paisley, UK)

Qiagen DNeasy Blood and Tissue kit (Crawley, West Sussex, UK)
Sample loading solution (SLS) (Beckman Coulter UK Ltd., Buckinghamshire, UK)
Sodium chloride (VWR International Ltd., Lutterworth, UK)
Sodium dodecyl sulphate (SDS) (VWR International Ltd., Lutterworth, UK)
Tris base (VWR International Ltd., Lutterworth, UK)
Tris chloride (VWR International Ltd., Lutterworth, UK)
TRIzol® reagent (Invitrogen, Paisley, UK)

2.2.2 Buffers

DNA lysis buffer: 20g DTAB (final concentration 8%), 22g NaCl (1.5M), 25mls of 1M Tris Cl pH 7.8 (100mM). Made up to 250mls with DDW.

Gel loading buffer: 3.9mls glycerol, 500µl 10% SDS, 25mg bromophenol blue, made up to 10mls with DDW.

TBE (10x): 108.9g Tris base, 55.7g Boric acid, 7.4g EDTA, made up to 1 litre with DDW.

2.2.3 DNA extraction

For some patients diagnosed prior to the start of the work in this thesis (August 2011), DNA was available in the UCLH DNA bank held within the Department of Haematology. These were predominantly patients that had consented to participate in one of the MRC/NCRI trials. Wherever possible for patients diagnosed prior to August 2011 and without stored DNA, archived bone marrow aspirate smears were obtained. DNA was extracted from material on these slides using the DNeasy Blood and Tissue kit (QIAGEN). Slides with coverslips were immersed in xylene for 24 hours to enable easier removal of the coverslip. For each patient sample, a total of 200µl PBS and 20µl proteinase K was placed on the slides (divided between slides if >1 slide used per patient) and a scalpel blade used to scrape the material into a 1.5ml Eppendorf tube. Samples were then processed according to manufacturer's instructions.

For patients diagnosed after August 2011, bone marrow aspirates that were surplus to diagnostic requirements were used to extract DNA. Samples were processed as described in section 2.1.4 but the extracted buffy coats were resuspended at a concentration of $1x10^6$ cells per 100µl 1x PBS. Two volumes of DNA lysis buffer were then added, mixed well and incubated at 68°C for 5 minutes. After cooling, the suspension was mixed well with an equal volume of chloroform before being centrifuged at 2000g for 20 minutes. Following this, the upper layer (containing the DNA) was added to an equal volume of 100% ethanol to precipitate the DNA. The DNA samples were then centrifuged at >8000g, washed twice with 70% ethanol, and then dissolved in DDW by end-over-end rotation overnight at 4°C.

2.2.4 Polymerase Chain Reaction (PCR)

The non-proof reading enzyme BIOTAQ™ DNA polymerase was used in combination with Bioline buffer and magnesium chloride according to manufacturer's recommendations. Forward and reverse primers were used at 0.5µM each, dNTPs at 200µM each, and approximately 50ng of genomic DNA template in a 20µl PCR reaction. PCR conditions included an initial denaturation step at 95°C for 1 minute, followed by successive cycles of denaturation (95°C for 30 seconds), annealing (30 seconds), and extension (72°C for 30 seconds), followed by a final extension of 72°C for 5 minutes. The annealing temperatures for the specific primer pairs are stated in the appropriate methods section of chapter 4.

2.2.5 Agarose gel electrophoresis

PCR products were run and visualised on 2% agarose gel. Gels were made by adding 2% weight/volume to 1x TBE. The agarose was melted by heating in a microwave oven. After cooling, ethidium bromide to a final concentration of 100ng/ml was added to the gel mixture that was then poured into a mould and allowed to set. To run samples, the gels were submerged in 1x TBE containing ethidium bromide to a final concentration of 100ng/ml. PCR product (4µl) was then added to 1µl loading dye and

loaded into a well within the gel. To confirm the size of the PCR products, for each gel, one well was loaded with 4µl of Hyperladder IV. The samples were electrophoresed at a constant current of 70mA for 30 minutes, the products were then visualised under a UV transilluminator and an image taken. The gels were then electrophoresed for a further 30 minutes before repeating the visualisation and imaging.

2.2.6 Fragment size separation using the Capillary Electrophoretic Genetic Analysis System (CEQ^{TM}) 8000

The CEQ^{TM} 8000 system was used to identify and quantify size differences between PCR products where the mutation resulted in the insertion of base pairs. Approximately 50ng of genomic DNA was amplified using the BIOTAQ^{TM} DNA polymerase conditions as described in section 2.2.4 but using 0.25µM of forward primer, 0.25µM of a fluorescently labeled reverse primer, and 25-30 PCR cycles.

CEQ^{TM} DNA Size Standard Kit 400 or 600 (3.5µl) was added to 1ml of SLS. Each PCR product (2µl) was added to 38µl SLS + CEQ^{TM} DNA size standard mix (or 36µl if 2 different PCR products were being assessed at the same time). The total 40µl was loaded onto the sample plate and one drop of mineral oil layered on top. The samples were then analysed by fragment separation on the CEQ^{TM} 8000 Genetic analysis system. Following separation of the PCR products according to size, the area under each peak was determined by the instruments' software algorithm, and the relative proportion of each peak determined by calculating the area under each peak as a percentage of total alleles. PCR products that had amplified sufficiently to saturate the fluorescent signal were rerun at a 1:10 dilution in PCR master mix. For PCR products with poor amplification, the PCR was repeated with an increased number of cycles up to a maximum of 30 cycles.

2.3 Flow cytometry

2.3.1. Reagents

Bovine Serum Albumin (Sigma Aldrich, Poole, UK)
Calcium chloride (VWR International Ltd., Lutterworth, UK)
Dulbecco's Phosphate Buffered Solution (PBS) without calcium/magnesium (PAA Laboratories Ltd, Somerset, UK)
Hydrochloric acid (1M) (Sigma Aldrich, Poole, UK)
Propidium iodide (Invitrogen, Paisley, UK)
Ribonuclease A (Sigma Aldrich, Poole, UK)
Tri-sodium citrate (dihydrate) (Sigma Aldrich, Poole, UK)
Triton X-100 (Sigma Aldrich, Poole, UK)
Tris base (VWR International Ltd., Lutterworth, UK)
Tris hydrochloride (Sigma Aldrich, Poole, UK)
Trypsin solution (2.5%) (Sigma Aldrich, Poole, UK)
Tween 20 (Sigma Aldrich, Poole, UK)

2.3.2 Buffers/Solutions

Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0): Tri-sodium citrate (dihydrate) 2.94g in 1 litre of DDW. Adjusted to pH 6.0 with 1M hydrochloric acid before adding 500µl Tween 20. Stored at 4°C.

Tris-HCl buffer (0.05M) containing calcium chloride (10mM): 6.06g Tris-HCl, 1.39g Tris base, 1.11g calcium chloride in 1 litre DDW.

CHAPTER 3. DEFINING THE CELL CYCLE IN AML BLASTS FROM BIOPSIES, ASPIRATES, AND PERIPHERAL BLOOD

3.1 Introduction

3.1.1 Relationship between cell cycle state and outcome in patients with AML

Numerous studies have sought to define the cell cycle status of leukaemic cells from patients with AML and relate this to clinical outcome (Table 3.1). The larger studies, those involving more than 100 patients, are discussed in greater detail.

Preisler and colleagues treated mononuclear cells harvested from patient BMAs with tritiated thymidine (^{3}HTdR) to label actively proliferating cells and expressed this as a relative proportion of total cells, *i.e.* the labelling index (LI) (Preisler *et al*, 1984). They found a significantly higher median LI for patients achieving CR compared to those with resistant disease for 39 patients that were treated with Ara-C alone (10.8% vs 6.8%, P=0.02), but not for 84 patients treated with Ara-C plus an anthracycline (9.6% vs 9%), nor for 26 patients treated with salvage regimens for relapsed disease (7.8% vs 6%). They concluded that the proportion of cells synthesising DNA at the time that treatment was initiated determined response to treatment, at least in part, only in those patients treated with Ara-C as a single agent. Of note, however, a high proportion of cycling cells did not guarantee sensitivity to Ara-C.

In a series of studies, Raza and colleagues investigated cell kinetics in more than 120 patients with "standard risk" AML, defined on the basis of age, comorbidities, and AML that was *de novo* (i.e. not related to treatment or a prior haematological disorder) (Raza *et al*, 1987; Raza *et al*, 1990; Raza *et al*, 1991). Using a double labelling technique, they sought to determine the length of S phase (Ts) and total duration of cell cycle time (Tc). Bromodeoxyuridine (BrdU), which is incorporated into newly synthesised DNA in place of thymidine, was infused into patients over one hour. BMAs (5ml) and bone marrow trephine biopsies (BMT) were then obtained. The LI of proliferating cells in the BMT was determined by staining with

Table 3.1 Summary of studies relating cell cycle status/kinetics to clinical outcome in patients with AML.

No. of patients	Patient characteristics	Source of cells	Technique to assess cell cycle	Main Findings	Reference
25		BMA	Pulsed cytophotometry	Lower % $S/G_2/M$ cells in AML than normal BM Patients with lowest $S/G_2/M$ did not respond to treatment	(Hillen *et al*, 1975)
51	Median age, 50 yrs	BMA	^{3}HTdR labelling *ex vivo*	Higher LIs associated with increased remission rates. Effect more pronounced in patients aged >40 yrs, no impact of LI in those ≤40 yrs. For patients <60 yrs, increased LI associated with shorter remission and decreased survival	(Hart *et al*, 1977)
41		BMA	^{3}HTdR labelling *ex vivo*	Higher LI in those responsive to treatment	(Zittoun *et al*, 1975)
23	Childhood AML, median 8 yrs (range, 0.1-16.4)	BMA	^{3}HTdR labelling *ex vivo*	No correlation between LI and achievement or duration of remission	(Murphy *et al*, 1977)
67	Previously untreated	BMA	^{3}HTdR labelling *ex vivo*	No correlation between LI and achievement or duration of remission	(Amadori *et al*, 1980)
78	56 previously untreated, 22 relapsed	BMA	Flow cytometry	Lower median % S phase cells in AML (8%) than ALL (10%), and normal BM (16%) Higher mean % S phase cells associated with lower circulating absolute blast counts %S phase cells did not predict for CR, length of remission, or OS	(Dosik *et al*, 1980)
61	38 previously untreated, 23 relapsed	BMT and BMA	Flow cytometry to define G_0/G_1, S, and G_2/M cells ^{3}HTdR labelling *ex vivo*	BMAs unreliable for determining cell cycle kinetics S-phase LI from biopsies not predictive of response to therapy	(Hiddemann *et al*, 1982a)
149	123 at presentation, 26 relapsed	BMA	^{3}HTdR labelling *ex vivo*	In newly diagnosed AML treated with Ara-C, higher LI associated with achieving remission No relationship between LI and remission in newly diagnosed or relapsed AML treated with anthracycline and Ara-C	(Preisler *et al*, 1984)
50	*De novo* Age range, 18-75 yrs	BMA	Flow cytometry	No difference in % S phase cells in those achieving CR versus not. Increased G_0/Early G_1 cells associated with increased rates of CR	(Ffrench *et al*, 1985)
47	Median 44 yrs (range, 18-78) 6 APL	BMA	Flow cytometry ^{3}HTdR labelling *ex vivo*	Higher number $S/G_2/M$ cells associated with increased rates of CR but shorter duration of longer-term responses	(Riccardi *et al*, 1986)

Table 3.1 (continued)					
73	Median 56 yrs (range, 22-81)	PB and BMA	Flow cytometry	Lower % S/G_2/M cells in PB associated with higher rates of CR and longer responses	(Guerci *et al*, 1989)
54	Standard risk AML	BMT and BMA	BrdU infused *in vivo*. BMT LI determined. BMA double labelled *ex vivo* with ^{3}HTdR. Length of S phase and total cell cycle time extrapolated from these	Increasing age associated with shorter S phase and total cell cycle time. No relationship between cell cycle parameters and response to induction Patients with slower cycling cells had longer remission	(Raza *et al*, 1990)
128 (54 from 1990 study)	Standard risk AML (≤70 yrs, no significant comorbidity, not sAML or tAML)	BMT and BMA	As for 1990 study	LI of BMT ≈3x higher than BMA with a positive but weak relationship. Total cell cycle time inversely related to BMT LI Remission longer for patients with higher BMA LI, longer length of S phase and higher total cell cycle time. No relationship between BMT LI and outcome	(Raza *et al*, 1991)
114	Median 54 yrs (range, 16-82) 38% Normal CGN 59% Abnormal CGN 3% Unknown	BMA	Cells cultured in serum- and cytokine-free media to assess autonomous proliferation. DNA synthesis assessed by incorporation of ^{3}HTdR	Higher rates of autonomous proliferation associated with inferior OS.	(Lowenberg *et al*, 1993)
204	*de novo* AML	126 PB, 78 BMA	Flow cytometry to define G_0/G_1, S, and G_2/M phases of cycle	No relationship between % S phase cells and response to induction therapy Increased % S phase cells associated with shorter survival	(Vidriales *et al*, 1995)
66	*de novo* AML	BMA	*Ex vivo* ^{3}HTdR incorporation following culture in GM-CSF	High LI associated with early response to treatment on day 10-16 BM	(Jahns-Streubel *et al*, 1995)
36	*de novo* AML	BMA	As for 1995 study	%S phase cells not associated with early clearance on day 5-7 marrow	(Jahns-Streubel *et al*, 1997)
226	181 *de novo* AML (27 = APL) 45 sAML	PB	Mononuclear cells plated on methylcellulose +/- supporting factors; autonomous growth and plating efficiency assessed	Highest autonomous growth in sAML post-MPN, lowest in APL CR not related to autonomous growth Higher proliferation associated with shorter survival	(del Canizo *et al*, 1998)
187	*de novo* AML: 25 favourable CGN 99 normal CGN 29 adverse CGN 34 other	BMA	*Ex vivo* ^{3}HTdR incorporation	Highest rates of proliferation in favourable CGN, lowest rates in adverse CGN Lower proliferation in APL than other favourable risk CGN but still higher than normal CGN	(Braess *et al*, 2001)

Table 3.1 (continued)					
91		BMA	*Ex vivo* ^{3}HTdR incorporation following culture in GM-CSF	Higher proliferation in favourable risk CGN compared to adverse risk CGN	(Jahns-Streubel *et al*, 2001)

Abbreviations: ^{3}HTdR, ^{3}H-thymidine; Abn, abnormality; AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; APL, acute promyelocytic leukaemia; Ara-C, cytarabine arabinoside; BM, bone marrow; BMA, bone marrow aspirate; BMT, bone marrow biopsy; BrdU, Bromodeoxyuridine; CGN, cytogenetics; CR, complete remission; GM-CSF, granulocyte-macrophage colony stimulating factor; LI, labelling index; MPN, myeloproliferative neoplasm; OS, overall survival; PB, peripheral blood; sAML, secondary AML; tAML, treatment related AML.

anti-BrdU antibodies. The BMAs were processed using Ficoll Hypaque centrifugation and low density cells (mononuclear cells) were collected. The cells were processed on ice and centrifuged at 4^{o}C "so that all metabolic activity in the cells was arrested prior to incubation in the second label". The harvested mononuclear cells were then cultured for one hour at a density of 5-10 million cells per ml in RPMI with 10% FCS in the presence of ^{3}HTdR, which was used as a second label of DNA synthesis. Ts was calculated using the formula Ts = (DL + ^{3}HTdR) x t/BrdU, where DL was the number of double-labelled cells, ^{3}HTdR and BrdU the percentage of positive cells for these labels, and t the time between the labels, which in this study was one hour. Tc was calculated using the formula Tc= Ts x GF/LI, where GF was the growth fraction and assumed to be 100%, and LI was the labelling index for BrdU of the trephine.

The authors found that there was a large variation between patients in values for Ts (range, 4-49 hours) and Tc (16-292 hours). The relationship between the LI of the BMT and the derived cell cycle parameters was inconsistent between studies, however in the report with the largest number of patients (n = 128), the LI of the BMTs was negatively correlated with Tc and there was a positive correlation between Tc and Ts (Raza *et al*, 1991). None of the cell cycle variables studied showed a statistically significant correlation with the attainment of CR following induction chemotherapy. The Tc was related to duration of remission, with patients with shorter Tc relapsing earlier, but there was no statistically significant correlation between remission duration and Ts. Furthermore, although patients with above median BrdU LI of BMAs had longer remission durations, this was not the case for the BrdU LI of the BMTs. The authors concluded that the most likely explanation for the observed relationship between remission duration of patients and some of the derived cell cycling kinetics of their leukaemic cells was that the cell cycle time played a role in determining the rate of leukaemic cell regrowth between courses of consolidation therapy and following the cessation of all therapy.

In these investigations by Raza and colleagues, the LI of BMTs was significantly higher than that of BMAs. In the first study, the mean was 23% vs 9% respectively, P=0.0003 (Raza *et al*, 1987). In a subsequent study (Raza *et al*, 1991), the mean and median LI (uptake of ^{3}HTdR) in BMTs from 122 patients were 25.1% and 25.0% respectively, whereas in BMAs from 114 patients the complementary results were only 8.8% and

8.0%. In the 111 patients in whom results from both BMT and BMA were available, the correlation was 0.379. Although statistically significant (P= 0.0001), this correlation is generally weak given that, in theory, these results were supposed to represent the same cells, taken at the same time, from the same patients and providing accurate information on cell cycle kinetics.

The study by Hiddemann(Hiddemann *et al*, 1982b) was a more thorough assessment of the discrepancy between values obtained from BMT and BMA samples. In a series of patients with either AML (n= 48) or other "non-leukaemic" disorders (n= 67), paired BMA and BMT samples were taken and assessed by flow cytometry for DNA (n=133). In over 70% of cases the results obtained from the biopsy were higher, with up to a 9.2-fold difference. In 48 patients where RNA content was assessed, similar results were obtained with up to an 8.1-fold change. When samples were assessed by autoradiography for the ^{3}HTdR LI (n=16), in all but one case results from the biopsy were higher, with a mean 1.82-fold difference and range up to 11.4 times higher. Results obtained from this technique and from flow cytometry were highly concordant ($r=0.94$, $P<0.001$). Very similar results were also obtained in a further 56 samples assessed by liquid scintillation counting using ^{3}HTdR uptake (counts per minute). In summary, results between the techniques used were highly concordant whereas results according to the source of blast cells showed significant differences.

The authors offered two potential explanations for these results: either a selective enrichment of proliferating cells during the biopsy preparation process, or a relative decrease in the proportion of proliferating cells in the BMA samples due to the admixture of non-proliferating blood cells. The former possibility was excluded given the high concordance between three different biopsy preparation techniques. To investigate the latter possibility, the authors mixed material from biopsies and blood and found that they gave similar results to the corresponding aspirates. This suggested that the higher proportion of cells in G_0/G_1 in the BMA samples was due to contamination with non-proliferating peripheral cells. Crucially, this happens even when small volumes of aspirate are taken as the aspirate volumes in this study were reported to be between 0.5 and 1ml. However, there was no conclusion as to the nature of the contaminating cells other than that they were "non-proliferating nucleated peripheral blood cells", although quantitative and functional differences in committed

granulocytic progenitors (CFU-C) and lymphocytes that have been reported with blood contamination were discussed.

Vidriales and colleagues assessed the prognostic value of the percentage of S phase cells, as assessed by flow cytometry of peripheral blood (PB) (n=126) or BMA samples (n=78) from 204 patients with *de novo* AML (Vidriales *et al*, 1995). The proportion of cells in S phase was only 2.3% in PB and 7.3% in the BMAs. The percentage of cells in S phase did not predict for response to intensive induction therapy, but increased numbers of cells in S phase were associated with shorter survival.

More recently, Braess and colleagues examined the proliferation of mononuclear cells from BMAs, as assessed by the incorporation of ^{3}HTdR into DNA in an *ex vivo* culture system (Braess *et al*, 2001). The highest and lowest proliferation rates were seen in the cells obtained from patients with favourable and adverse karyotypes respectively. Higher rates of proliferation correlated with improved outcome within the intermediate and adverse risk groups but not the favourable risk group. Variations in proliferation between patients within the favourable group did not affect outcome. APL patients had the lowest proliferation rates of the favourable groups, however this was still higher than the intermediate or adverse risk groups. The authors proposed that differences in proliferative activity are a characteristic of cytogenetically defined subgroups and that this may be related to the relative cytoreductive effects of Ara-C based induction on cells with different karyotypes.

Other studies have assessed autonomous growth, *i.e.* cells growing in serum or cytokine-free media, as a means to try and infer cell cycle kinetics. Lowenberg and colleagues assessed autonomous growth in the cells taken from BMAs of 114 patients (Lowenberg *et al*, 1993). The rate of DNA synthesis was assessed by the incorporation of ^{3}HTdR and three proliferative groups (high, intermediate, and low) were defined. There were no statistically significant correlations between the rate of autonomous proliferation and age, sex, WCC, percentage of leukaemic blasts in the marrow, or the number of cytogenetic abnormalities, although it was noted that all patients with t(8;21) had low levels of autonomous proliferation. Patients with M4 or M5 disease had higher rates of autonomous proliferation. All patients were included in a survival analysis; 91 received anthracycline + Ara-C induction therapy. In multivariate analysis, autonomous

proliferation was an independent prognostic factor for OS at 3 years (low growth 36% vs high growth 3%, P<0.0001), and rates of CR also varied with proliferation group (low 68%, intermediate 62%, high 39%, P=0.04).

A later study of 226 patients (del Canizo *et al*, 1998) examined PB-derived mononuclear cells plated in methylcellulose with and without stimulating factors to determine autonomous growth and plating efficiency. These were then used to derive an "autonomous proliferation index". AML secondary to a myeloproliferative neoplasm had the highest rates of autonomous growth and proliferation index, whereas patients with M3/APL had the lowest autonomous growth but the highest plating efficiency. There was no correlation between autonomous growth or the derived index and rates of CR, but a higher proliferation index correlated with shorter survival.

In summary, the results of previous investigations relating some of the cell cycle dynamics to clinical outcome in patients with AML are inconsistent, and in some cases the conclusions are diametrically opposed. Increased proliferation, or increased numbers of S-phase cells as a surrogate for this, has been found to be favourable, adverse, or of no prognostic value in predicting response to treatment, time to relapse, or overall outcome.

3.1.2 Limitations of previous studies

The conflicting results seen in these studies of cell cycle parameters in patients with AML are likely to reflect a number of established or potential differences between the studies.

3.1.2.1 Differences in source of leukaemic cells

The above studies show clear discrepancies in the average number of cells in S phase between BMTs, BMAs, and PB, with the highest values seen in BMTs and the lowest in PB. Using paired samples, Hiddemann and colleagues demonstrated a lower number of S phase cells in BMAs than BMTs (Hiddemann *et al*, 1982a). The subsequent studies

by Raza and colleagues corroborated the higher LI seen in BMTs than BMAs (Raza *et al*, 1990). The flow cytometric studies by Vidriales (Vidriales *et al*, 1995) showed higher average results for BMAs than PB, although the samples were not paired. Other studies using PB have shown a consistently lower proportion of S phase cells or LI by ^{3}H-thymidine incorporation than comparable studies using BMA samples. These lower values for BMAs and PB as compared to BMT samples have been ascribed to 'contamination' by non-proliferating nucleated peripheral cells (Hiddemann *et al*, 1982b), although without firm conclusions as to whether these are leukaemic blasts or other mononuclear cell types.

3.1.2.2 Difference in techniques used to assess the cell cycle

There are a number of differences between the studies in the techniques used to process, measure, and analyse the cell cycle profile or kinetics.

i. Measurement of S phase cells by incorporation of ^{3}HTdR or BrdU as compared to measurement of S/G_2/M cells by flow cytometry.
ii. *In vivo* labelling as compared to *ex vivo* labelling following various lengths and types of processing or culture. Such *ex vivo* growth during processing and/or subsequent culture is unlikely to represent the true nature of cell kinetics within the bone marrow microenvironment and under physiological cytokine stimuli.
iii. Use of autonomous growth as opposed to measuring *in vivo* kinetics or kinetics with serum or cytokine support. This is more a measure of cell survival and resistance to apoptosis than of proliferation and, again, is unlikely to be representative of the cells within the bone marrow niche.
iv. Exclusion of cases with lower levels of viable cells following processing. For example, Braess *et al* (2001) only included samples with >95% viability following processing.
v. Assumptions used to derive formulae for inferring cell cycle kinetics. For example, the studies by Raza and colleagues assumed that the rate of cell export from the bone marrow equals that of cell production, and that cells are exported exclusively in G_1. Furthermore, they assumed that there is a "steady state" rather than exponential growth in the *ex vivo* culture and a 100% proliferation fraction in BMTs.

3.1.2.3 Patient-, disease-, and treatment-related factors

The patient-, disease-, and treatment-related factors vary between studies.

i. In some studies there is limited information on patient age. This may be of importance given the difference in disease biology with age. The increased expression of, for example, drug efflux pumps with age may render cells resistant to cell cycle targeting agents regardless of cell cycle status. One study has suggested a difference in the impact of cell cycle status depending on the age group examined (Hart *et al*, 1977).

ii. Earlier studies simply refer to acute non-lymphocytic leukaemia. There is limited information in some studies as to whether the AML was primary or secondary, initial presentation or relapse.

iii. There is limited information on FAB types such that there is likely to be differences between studies in, for example, the number of patients included with M3/APL. This could have a large impact, particularly if patient numbers are small. Nevertheless, there is controversy even for cell cycle status of M3/APL. Raza (Raza *et al*, 1991) found that M3/APL subtypes were associated with slower cycling rates. In contrast, (Braess *et al*, 2001) found that the APL subtype was cycling faster than all other leukaemia subtypes bar the core-binding factor leukaemias.

iv. Treatments in some of the earlier studies pre-date the use of the standard remission induction of anthracycline + Ara-C "3+7". Furthermore, not all studies contained consolidation treatment for patients achieving remission. Nevertheless, conflicting results have also been reported between studies in which patients received what is now accepted to be standard induction therapy followed by remission consolidation.

v. There is limited information on patient's cytogenetics or risk-group. This may be important in view of the findings by (Braess *et al*, 2001) and (Jahns-Streubel *et al*, 2001) that different cytogenetic risk groups appear to have different measured cell-cycle parameters.

vi. Some studies excluded patients with a marrow blast count below a certain threshold. (Braess *et al*, 2001) excluded cases with ≤70% bone marrow infiltration, and the studies

by (Dosik *et al*, 1980) and (Ffrench *et al*, 1985) required ≥70% and >80% marrow involvement respectively for a patient to be included in the cohort. This may select for *de novo* AML over secondary AML, which is likely to impact on cell cycle status, particularly in view of the different spectra of mutations associated with these entities (Lindsley *et al*, 2015).

vii. All but three of the studies predate the discovery of *FLT3*-ITDs in AML, and all of the studies predate the identification of mutations in the *NPM1* gene, disease-related molecular mutations now known to have a significant biological and prognostic impact.

In summary, the discrepancies reported in the prognostic impact of cell cycle analysis are likely to reflect a combination of the above features. Perhaps one of the most fundamental features here is the potential difference introduced by the source of the blast cells. Despite the concerns raised by (Hiddemann *et al*, 1982b) about the variable impact of contamination of non-cycling cells in BMA samples, few studies have looked at outcome data where only BMT results have been considered, and such studies that do exist have only looked at LI as defined by the percentage of cells incorporating ^{3}H-thymidine. There is a need therefore to assess cycle status in the *in vivo* state, without blood cell contamination.

A robust method for identifying cells in different stages of the cell cycle using IHC was described in Chapter 1, where expression of MCM2 can identify cells in cycle (not G_0), MIB-1 (detecting MKI67) identifies actively proliferating cells, and geminin identifies cell that have progressed through the G_1/S phase checkpoint (i.e. cells in S/G_2/M phases). These cell cycle markers have not been used in aggregate to define the cell cycle status in patients with AML.

3.1.3 Aims of this chapter

Using these established cell cycle markers, the aims of the studies presented in this chapter were:

1. To define the cell cycle state in PB, BMA, and BMT samples from patients with AML.

2. To assess potential differences in measured cell cycle states from the different sources of sample.
3. To assess the potential impact of PB contamination on the results of cell cycle analysis.
4. To assess the potential impact of these differences on biological studies.

3.2 Materials and methods

3.2.1 Patient samples

Pre-treatment BMT and BMA samples, taken from the posterior superior iliac crest, and PB samples were collected from 8 patients with a diagnosis of AML. In 7 patients this was at initial presentation and in one patient (patient 5, Table 3.2) samples were taken at relapse prior to salvage therapy. The UCL/UCLH Research Ethics Committee approved the studies. Informed consent was obtained according to the Declaration of Helsinki.

3.2.2 Processing of Patient Samples

The BMTs were decalcified, formalin-fixed and paraffin-embedded in the UCLH core pathology facility using standard methods. For BMA samples, the first 2.5ml was used for diagnostic morphology, immunophenotyping, and cytogenetics. A further 5mls was processed for flow cytometry and cell cycle analysis. At the same time, 5mls of paired PB was processed. Samples were centrifuged at 600g for 5 minutes and buffy coats were collected. Samples were then resuspended, centrifuged for a further 5 minutes at 600g before plasma was removed and discarded, and the residual buffy coat collected and pooled with the first buffy coat collection.

Cells were resuspended in 1x PBS up to a total volume of 2ml. A 1x ammonium chloride red cell lysis buffer (final concentration 0.8%) was prepared by making a 1 in 10 dilution of 10x stock solution in DDW. Four volumes of this red cell lysis buffer (8mls) were added to the cell suspension, mixed well and incubated on ice for 10 minutes. The cells were then pelleted at 400g and resuspended in 1x phosphate-buffered saline (PBS).

Trypan blue exclusion was used to determine the number of viable cells in a suspension. In contrast to non-viable cells, viable cells with an intact cell membrane are able to exclude trypan blue. Thus, when visualised using light microscopy, viable cells (clear cytoplasm) can be readily distinguished from non-viable cells (blue cytoplasm). An aliquot of the cell suspension (10µl) was mixed with an equal volume of 0.4% trypan blue and transferred to a haemocytometer where the number of viable and non-viable cells were counted under a light microscope.

Cells were washed in 1x PBS and pelleted by centrifugation at 400g before being re-suspended and fixed in 10% neutral-buffered formalin ($1x10^6$ viable cells/ml). An aliquot was stored at -20°C until required for the flow cytometry experiments; in addition $1x10^6$ cells were pelleted at 400g, re-suspended in 1ml 2% agarose (weight/volume in 1x PBS) pre-warmed to 60°C, centrifuged at 1500g for 20 seconds and then the agarose allowed to set. The agarose plugs were subsequently formalin-fixed and paraffin-embedded in the UCLH core pathology facility using standard methods.

3.2.3 Immunohistochemistry (IHC) and immunocytochemistry (ICC)

Sections of 3µm thickness were cut from each formalin-fixed paraffin-embedded biopsy (by the UCLH pathology core) and pellet (by Miss Ayse Akarca, Research Department of Haematology, UCL) and transferred to glass microscopy slides. Tonsil tissue containing both glandular and epithelial components was processed at the same time and used as a positive control. Sections were immuno-stained using Leica Bond

Polymer Refine Detection and the Bond–Max^{TM} automated system (Leica Biosystems, Newcastle, UK).

Sections were immunostained using the Bond^{TM} Polymer Refine Detection kit and Bond^{TM}–Max automated system (Leica Biosytems, Newcastle, UK). Within the automated system, the tissues sections were dewaxed and then heated to 100°C for the purposes of antigen retrieval. They were incubated with peroxide block for 5 minutes, washed 3 times and then incubated for 40 minutes with the respective primary antibody. Primary antibodies were used at the following concentrations: MCM2 (1:3000), Geminin (1:200), MIB-1 (1:90), and Cleaved Caspase 3 (1:300). They were then washed 3 times before incubation with rabbit anti-mouse antibody linked to horseradish peroxidase. Following a further wash step, the sections were incubated with goat anti-rabbit antibody linked to horseradish peroxidase. Following further washes, the sections were incubated for 5 minutes with 3-3'-diaminobenzidine (DAB) enhancer before a 2-minute incubation with haematoxylin counterstain and a final wash step. Cut section of paraffin-embedded tonsil tissue containing both glandular and epithelial components was processed at the same time and used as a positive control.

The stained sections were washed for 15 minutes in DDW and then dehydrated in ethanol. The ethanol was then removed in a series of xylene baths before applying coverslips. Following IHC/ICC staining, the sections were examined microscopically to check for efficient staining.

The sections were examined at 40X magnification. For each section a minimum of 200 blasts were assessed and LIs determined based on the presence of nuclear staining (number of positive cells as a percentage of the total number of positive and negative cells).

3.2.4 Flow cytometry

3.2.4.1 Preparation of cell suspension from BMTs

Cells from paraffin-embedded BMTs were prepared for flow cytometric analysis from 50μm sections (cut by Miss Ayse Akarca, Research Department of Haematology, UCL) using a protocol adapted from previously published methods (Jordanova *et al*, 2003). Sections were dewaxed 3 times for 5 minutes in 2mls xylene and rehydrated in ethanol baths of descending concentration. After washing twice in 2mls 1X PBS, the sections were immersed in 2mls cold citrate solution (10mM, pH 6.0) and incubated for 60 minutes at 80°C. Sections were then cooled to room temperature and pelleted by centrifugation at 400g. Pellets were digested for 10 minutes at 37°C in 2mls 0.1% trypsin solution in 0.05M Tris-HCl buffer containing 10mM calcium chloride. Digestion was then blocked with 1X PBS containing 1% bovine serum albumin. Following mechanical mincing by drawing the suspension through needles of decreasing gauge down to 27G, samples were filtered through 70μm then 40μm mesh nylon cell filters. Samples were then washed in 1X PBS and processed for cell cycle analysis by flow cytometry in parallel with BMA and PB samples.

3.2.4.2 Cell cycle analysis by flow cytometry

For BMT samples, all harvested cells were pelleted, and for BMA and PB samples, $5x10^5$ formalin-fixed cells were pelleted at 400g. Cells were re-suspended in 1ml of a 1:1 mix of cold PBS and 0.1% Triton X-100 in PBS for 3 minutes at room temperature, washed in PBS containing 0.1% bovine serum albumin and re-suspended in 500μl PBS containing 0.1% ribonuclease A and 5μL 2.5mg/μl propidium iodide. Cells were analysed on a BD Accuri^{TM} C6 cytometer (BD Biosciences, New Jersey, USA). Blast cells were gated on the basis of forward and side scatter (FSC) and doublets excluded following plotting of FSC-A (area) against FSC-H (height). Cells were acquired through the flow cytometer at a rate of between 10 and 20 cell events per second. At least 500 events per sample were collected. DNA content was assessed on all gated cells in the FL2 channel (488nm laser, filter 585/40) to determine the percentage of blast cells in the $S/G_2/M$ phases of the cell cycle ($\geq$2n).

3.2.5 RNA expression profiling of BMT, BMA and PB cells

RNA was extracted from BMT, BMA, and PB samples collected from a single patient and processed within 30 minutes of collection. The biopsies were teased apart using a 27G needle, suspended in RPMI containing 10% fetal calf serum and filtered through a 40µm cell strainer. The cells were pelleted at 400g, washed in cold 1X PBS, then suspended in 100µl TRIzol® (Life Technologies Ltd, Paisley, UK) and stored at -80^{o}C until required. Buffy coats were harvested from the BMA and PB samples and the red cells lysed using 0.8% ammonium chloride. The remaining cells were washed, counted, pelleted, then suspended in 100µl Trizol per 1x10^{6} cells and stored at -80^{o}C. Samples were thawed on ice and processed using the Purelink^{TM} RNA Micro Kit with the addition of Purelink^{TM} DNase/Carrier RNA (Life Technologies) according to manufacturer's instructions. RNA quality was checked using a micro capillary chip (RNA 6000 Nano) for the Agilent Bioanalyser 2100 (Agilent, Santa Clara, California, United States) according to manufacturer's instructions.

The RNA-seq analysis was performed with help and guidance from Dr Tony Brooks and Dr Mike Hubank, UCL Genomics, UCL Institute of Child Health. RNA was prepared for sequencing following the Illumina Truseq protocol, multiplexed, and sequenced (43bp paired-end) on an Illumina NextSeq 500 sequencer (Illumina, San Diego, California, United States) to yield approximately 15 million reads per sample. De-multiplexed FastQ data were analysed at UCL Genomics. Sequencing reads were aligned to reference genome GRCh38 using tophat v2.0.13. When using paired end data, PCR duplicates were removed using Picard tools (v1.100). GTF (Gene transfer format) files describing gene features were obtained from the Ensembl website (http://www.ensembl.org/info/data/ftp/index.html) and read count data for each genomic feature were obtained using the python scripts provided as part of the Deseq2 package. Read count data were subsequently normalised and differential expression was computed using the Deseq2 procedure. Normalised read counts (normalised using the Deseq2 library size procedure) were generated for visualisation in GeneSpring 13.0 (Agilent Technologies). The set of scripts used are freely available at https://github.com/plagnollab/RNASeq_pipeline.

Analysis was performed using GeneSpring wih training and guidance in use of the program provided by Dr Mike Hubank. For the purposes of analysis, and to try and minimise the impact of stromal contamination, only genes with Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values ≥20 in both the BMT and PB samples were included. The proposed gene functions were assessed using data available from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/gene), and genes coding for proteins with predominantly stromal roles were also excluded from the analysis.

3.2.6 Statistical analysis

Statistical analysis was performed using Graphpad Prism software version 6 (GraphPad Software Inc., California, USA). LIs and derived ratios for BMT, BMA, and PB samples were compared using the 2-tailed Wilcoxon matched-pairs signed rank test. Comparisons across all three tissue compartments simultaneously was performed using the Friedman test. Correlations between results for BMT, BMA, and PB samples were assessed using Spearman's rank correlation. Correlations between % $S/G_2/M$ results obtained by IHC for geminin and flow cytometry using propidium iodide were performed using Spearman's rank correlation. Agreement between the two techniques for measurement was assessed by Bland-Altman plot. A P value <0.05 was considered significant.

3.3 Results

3.3.1 Cell cycle analysis in matched BMT, BMA and PB samples

3.3.1.1 Patient characteristics

The demographics of the eight patients whose cells were used for cell cycle analysis is shown in Table 3.2. The median age was 64 years (range, 34-74) and median peripheral

WCC at presentation was $27.5x10^9$/l (range, 2-365). Four patients had a normal karyotype, two had a monosomal karyotype, one a complex karyotype, and no cytogenetic data was available for the patient that was assessed at relapse.

Table 3.2 Demographics and presenting features of the 8 AML patients used in the matched sample analysis

Case	Age, yrs	Sex	WCC ($x10^9$/l)	Cytogenetics
1	62	M	141	Normal
2	72	F	13	Normal
3	60	F	131	Monosomal
4	74	M	2	Complex
5	70	M	4	Not available
6	34	F	7	Normal
7	65	F	42	Monosomal
8	63	M	365	Normal

3.3.1.2 Immunohistochemistry (IHC) and Immunocytochemistry (ICC)

PB, BMA, and BMT samples from each patient were stained with antibodies against MCM2, MIB-1, and geminin, and LIs were determined for the percentage of positive blast cells as a proportion of total blast cells. Clear positive and negative cells were evident and representative images are shown in Figure 3.1.

There was no significant difference between BMT, BMA, and PB samples in the proportion of blasts expressing MCM2 (medians 95.5%, 93.5%, and 94% respectively, P=0.21 for Friedman test of data to determine differences across all tissue types) (Figure 3.2A). For paired comparisons of BMT vs BMA, BMT vs PB, and BMA vs PB, the values using a Wilcoxon matched-pairs signed rank test were P=0.28, P=0.17, and P=0.99 respectively, indicating that nearly all the blast cells were non-quiescent and in-cycle, irrespective of location. The percentage of blasts in-cycle was also highly correlated between BMT and BMA samples (r=0.97, P=0.0006) (Figure 3.2B), BMT and PB samples (r=0.98, P=0.0004) (Figure 3.2C), and BMA and PB samples (r=0.95, P=0.001) (Figure 3.2D). There was, however, a significant difference in the proportion of blasts expressing MIB-1, median LI 63% for BMT versus 4% for PB samples

(P=0.008) (Figure 3.3A), indicating that the BMT blasts had a very high proliferative fraction whereas, in general, PB blasts were not actively proliferating. There was a non-significant correlation between the MIB-1 LI found in BMT as compared to BMA samples (r=0.6, P=0.1) (Figure 3.3B), and no correlation between the MIB-1 LI in PB and either BMT samples (r=-0.07, P=0.9) (Figure 3.3C), or BMA samples (r=-0.11, P=0.8) (Figure 3.3D).

In accord with this, the median geminin LI indicating progression beyond G_1 was 21% for BMT versus 1% for PB samples (P=0.008) (Figure 3.4A). The median for BMA samples was 6%, and was significantly different from both BMT samples (P=0.008) and PB samples (P=0.008). The correlation between geminin LI in BMT and BMA samples was weak and non-significant (r=0.42, P=0.3) (Figure 3.4B). There was no significant correlation in the geminin LI between PB samples and either BMT samples (r=0.19, P=0.7) (Figure 3.4C) or BMA samples (r=-0.27, P=0.3) (Figure 3.4D).

For the derived MIB-1/MCM2 ratio, there were significant differences between blast cells obtained from BMT, BMA, and PB samples (medians 0.65, 0.24, and 0.035 respectively, Friedman test for differences across the sample types P<0.0001, individual comparisons BMT vs BMA, BMT vs PB, and BMA vs PB, all P=0.008) (Figure 3.5A). There was a trend for a correlation between values obtained from BMT and BMA samples (r=0.7, P=0.06) (Figure 3.5B). There was no significant correlation between values obtained from PB and either BMT samples (r=-0.19, P=0.6) (Figure 3.5C), or BMA samples (r=-0.25, P=0.5) (Figure 3.5D).

The derived geminin/MIB-1 ratio did not significantly differ between the groups, although for some patients there were clearly large differences between the tissue compartments (Figure 3.6A). For example, for one patient (patient 4, Table 3.2), the geminin/MIB-1 ratios obtained for the BMT, BMA, and PB samples were 0.09, 0.07, and 0.5 respectively, and for another patient (patient 8, Table 3.2) they were 0.59, 0.23, and 0.13 respectively. None of the correlations for this ratio between the different sources of blast cells reached statistical significance (Figures 3.6B-D).

Figure 3.1 Detection of cell cycle proteins using immunohistochemistry. Representative images of immunohistochemical and immumocytochemical staining for (A) MCM2, (B) MIB-1, and (C) geminin in control tonsil tissue and bone marrow biopsy (BMT), bone marrow aspirate (BMA), and peripheral blood (PB) samples from an AML patient. Images are shown at x20 magnification.

Figure 3.2 Comparison of MCM2 LIs in BMT, BMA, and PB samples taken from the 8 patients.

Removed for Copyright

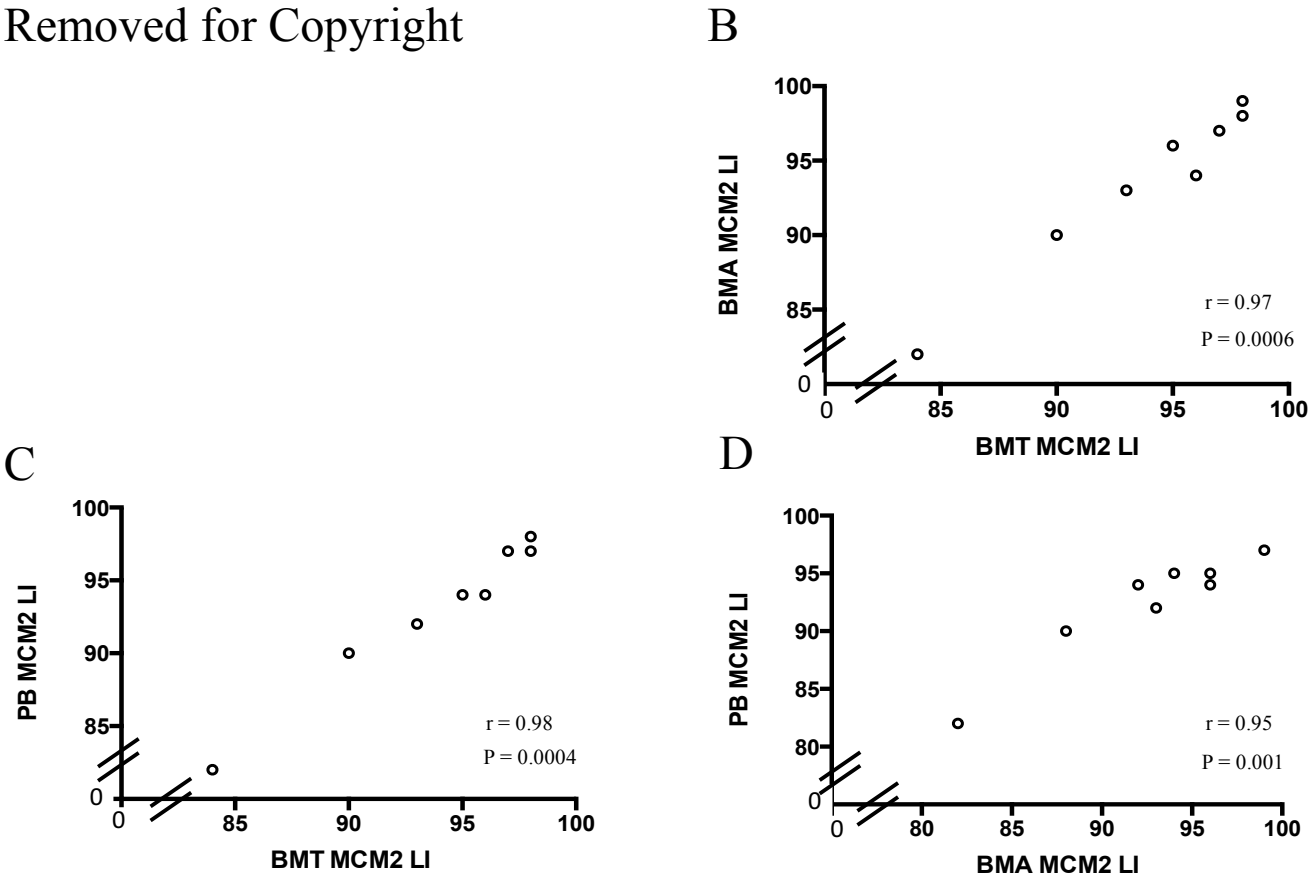
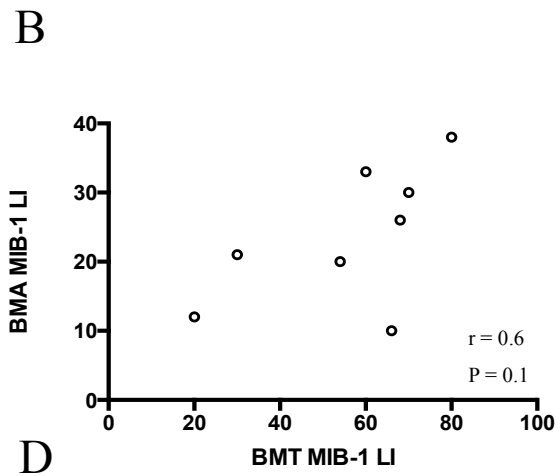
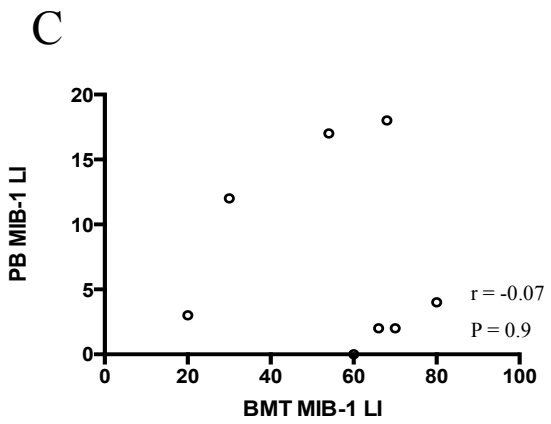


Figure 3.3 Comparison of MIB-1 LIs in BMT, BMA, and PB samples taken from the 8 patients

Removed for Copyright





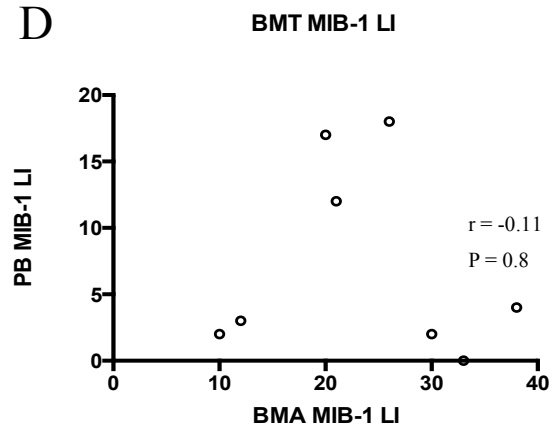


Figure 3.4 Comparison of geminin LIs in BMT, BMA, and PB samples taken from the 8 patients.

Removed for Copyright

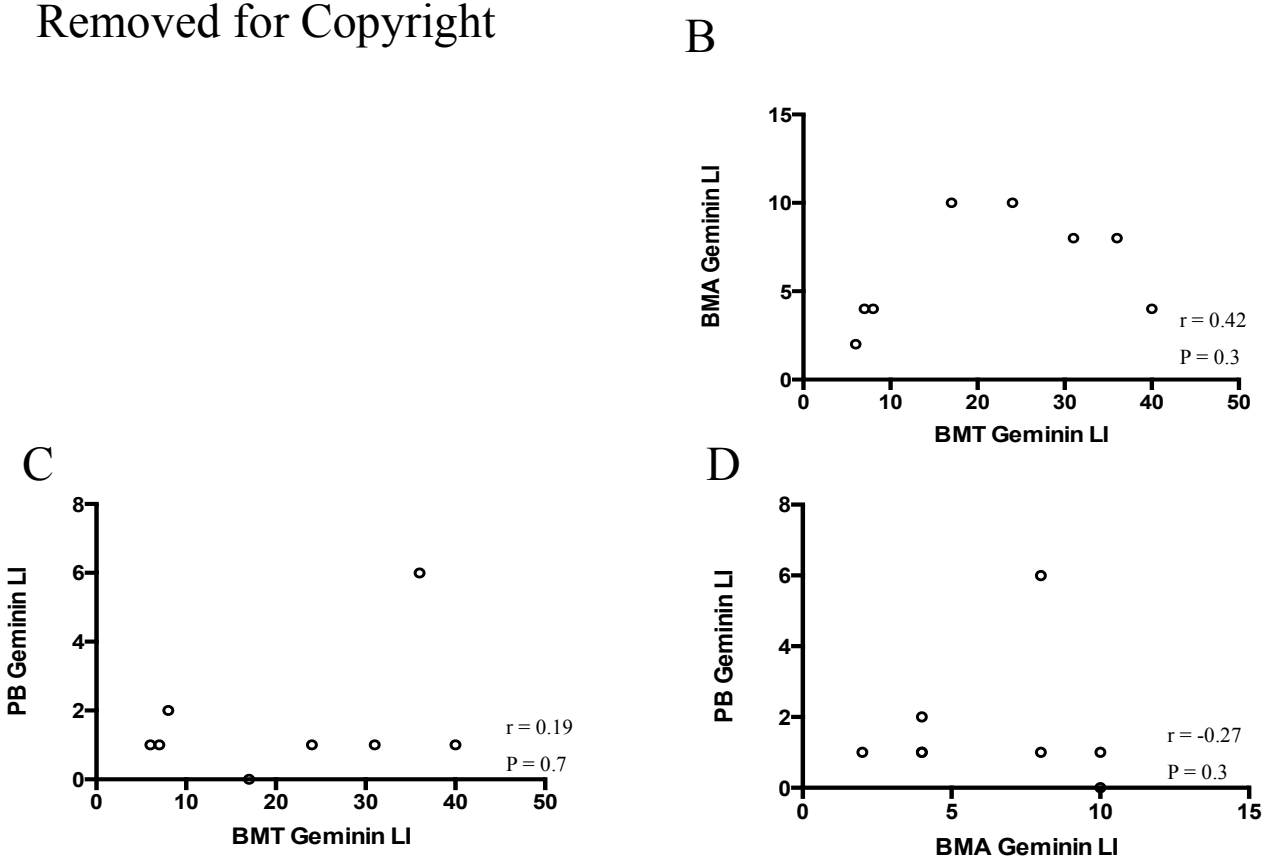


Figure 3.5 Comparison of the MIB-1/MCM2 ratios in BMT, BMA, and PB samples taken from the 8 patients.

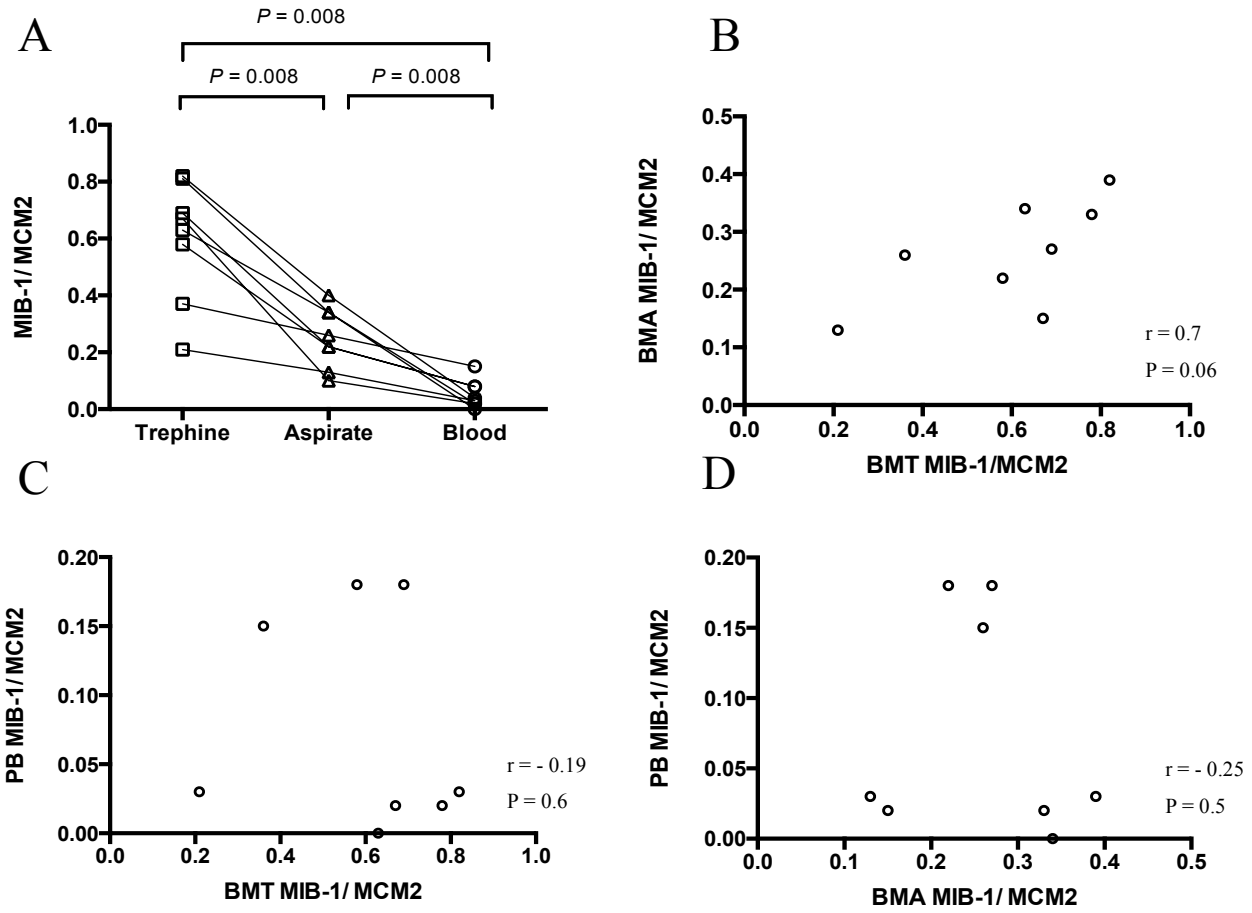
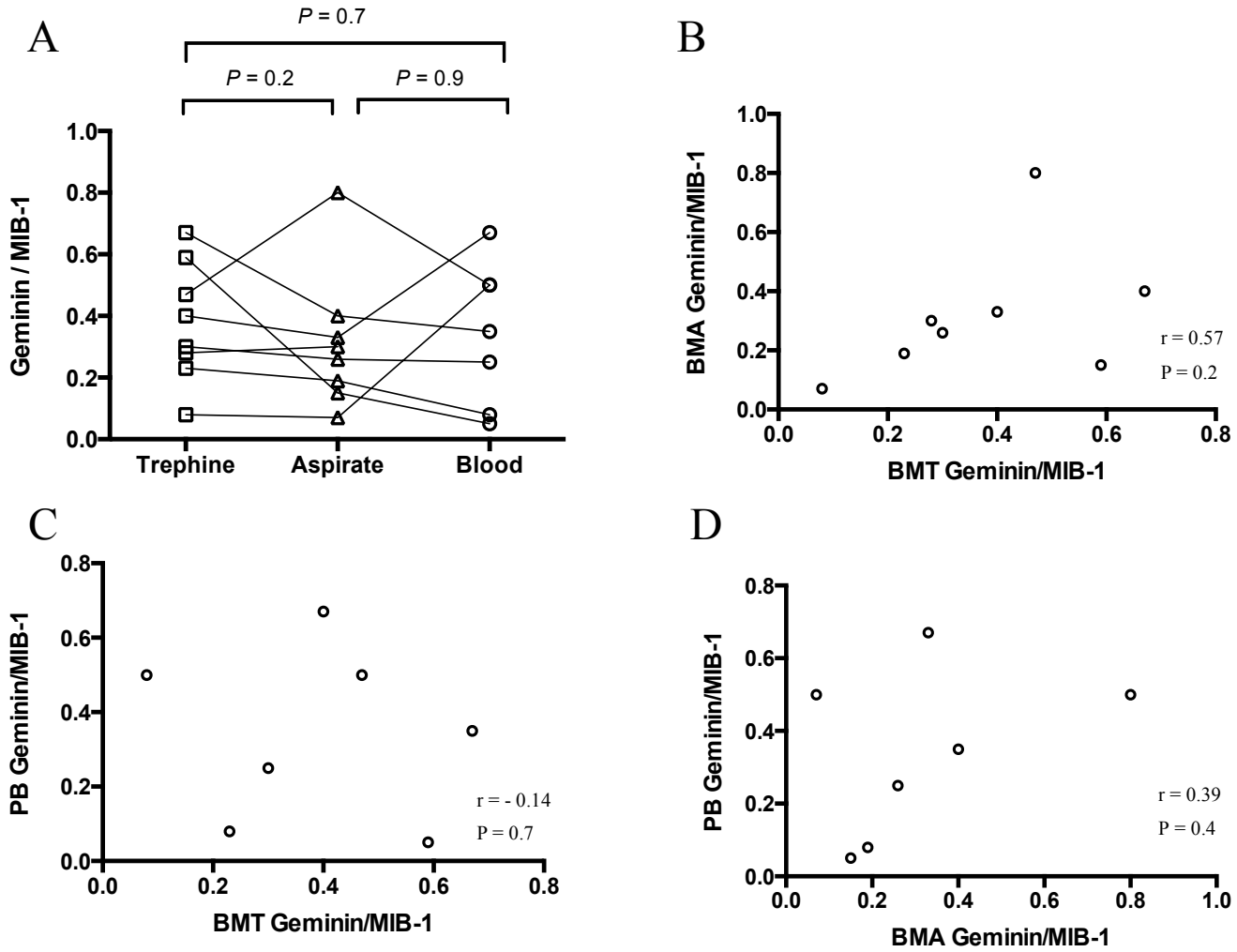


Figure 3.6 Comparison of the Geminin/MIB-1 ratios in BMT, BMA, and PB samples taken from the 8 patients.



3.3.1.3 Flow cytometry

Samples from each source were also assessed using flow cytometry with propidium iodide and the percentage of blast cells in $S/G_2/M$ determined. Representative plots from one patient (patient 8, Table 3.2) are shown in Figure 3.7A. There was a good correlation between the proportion of propidium iodide-stained cells in $S/G_2/M$ and the geminin LI obtained from IHC/ICC, irrespective of sample source ($r=.986$, $P<0.0001$) (Figure 3.7B). Bland-Altman plots showing the difference between the two results for all samples and for the 3 tissue compartments assessed separately further indicate that there was minimal difference between the results (Figure 3.8.A-D, Table 3.3), and indicate that there was no apparent bias for either method.

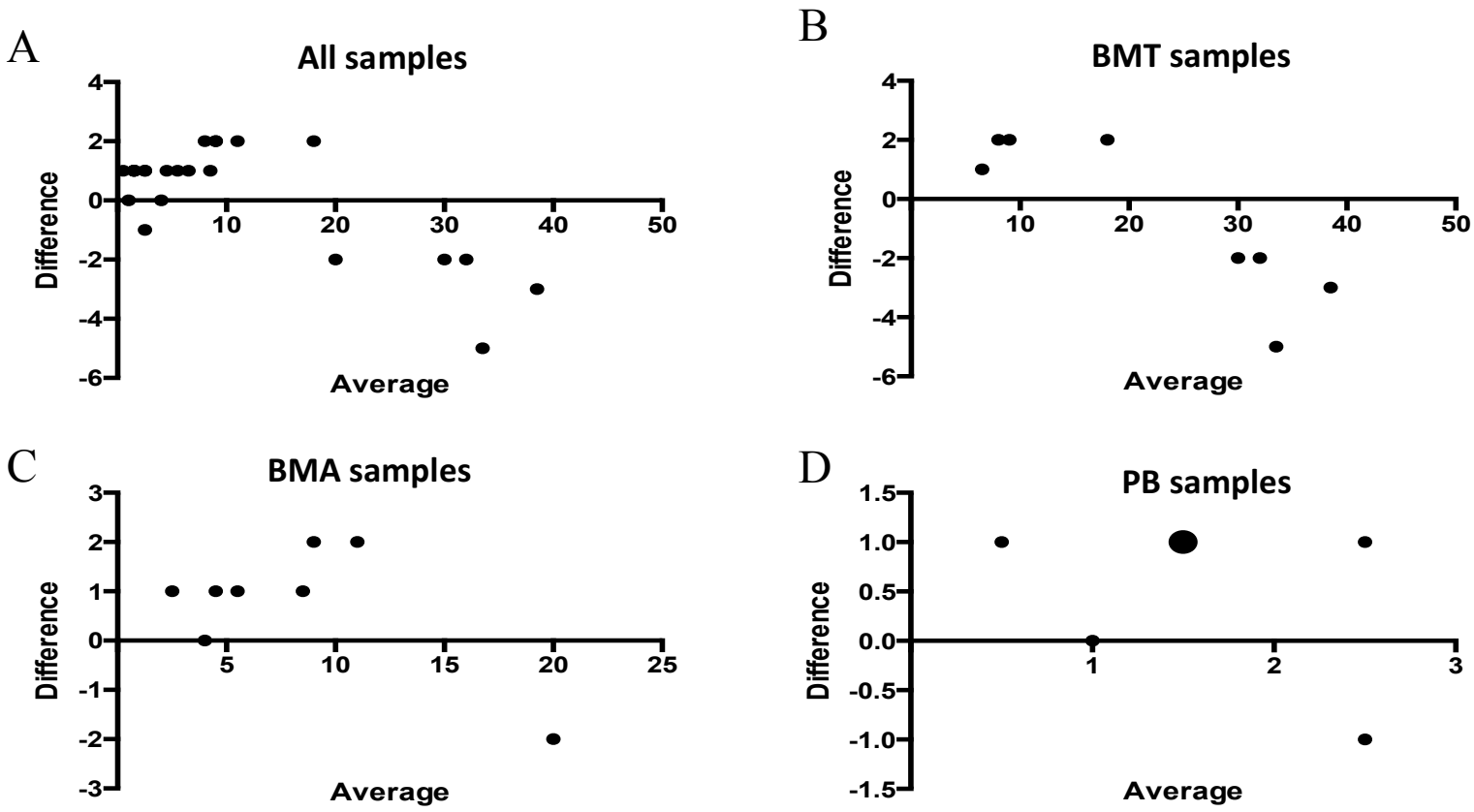
Table 3.3 Results of the Bland Altman analysis for agreement between % $S/G_2/M$ cells determined by immunohistochemistry/immunocytochemistry (geminin-positive) and by flow cytometry using propidium iodide.

	All tissue compartments	BMT	BMA	PB
Bias	0.25	-0.625	0.75	0.625
Standard deviation of bias	1.82	2.72	1.382	0.744
95% limits of agreement	-3.325 - 3.825	-5.961 - 4.711	-1.762 - 3.262	-0.833 - 3.083

Removed for Copyright

Figure 3.7 Cell cycle analysis using flow cytometry and correlation with IHC/ICC results. (A) Representative examples of propidium iodide staining to assess cell-cycle status in blast cells from BMT, BMA, and PB samples from one patient (patient 8). (B) Correlation between cell-cycle analysis results obtained using flow cytometry and IHC/ICC.

Figure 3.8 Bland-Altman plots comparing the proportion of geminin-positive by IHC/ICC and in S/G_2/M phase by flow cytometry. Difference refers to the difference between the results of the same sample using the two methods (flow cytometry – IHC/ICC) and average to the mean of the results for the two methods. The large filled circle in figure D denotes 4 samples, all of which have the same position in this Bland-Altman plot.

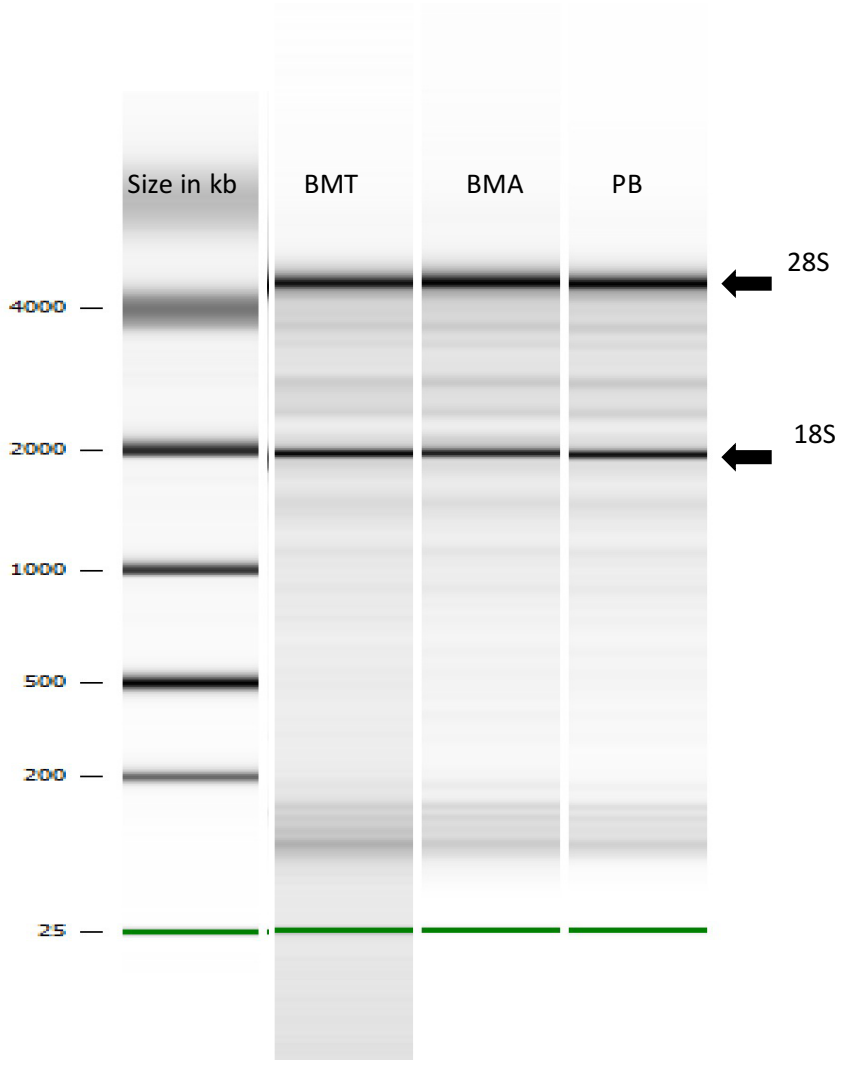


3.3.2 Comparison of PB, BMA, and BMT samples using gene expression profiling

RNA was prepared from BMT, BMA, and PB samples from one patient for gene expression profiling. The patient had a presenting WCC of $98x10^9$/l and >90% blasts in all three samples, precluding any need for blast cell purification. This was important as seven previous attempts to do RNA comparisons between BMT, BMA, and PB samples from patients where blast numbers were low in the PB had resulted in the RNA signature being heavily influenced by the differences in tissues in terms of cell type composition. For example, larger numbers of lymphocytes in PB made true differences in the RNA signature from blasts difficult to assess as it was essentially a comparison of a blast/lymphocyte mixture versus purer blast population. Samples were processed within 30 minutes of collection. Good quality RNA was obtained from all samples, as assessed by analysis on an Agilent Bioanalyser 2100 (Figure 3.9). cDNA libraries were prepared and sequenced as described above, and the data was analysed using GeneSpring 13.0.

A concern for analysis of the data was the likely impact of contaminating stromal tissue in the BMT sample that would influence the results. In an attempt to minimise this impact, two further approaches were taken. Firstly, genes with FPKM values <20 in the PB sample were excluded. This was partly to focus on more highly expressed genes but also an attempt to exclude major stromal genes that would be unlikely to be expressed at high levels in the peripheral blood. The value of 20 was chosen as an approximation of the median FPKM value as the median FPKM values in the BMT, BMA, and PB samples were 24.5, 19.9, and 22.7 respectively. As an illustration of this approach, *COL1A1,* which codes for the alpha chain of type 1collagen and is highly expressed in connective tissue, was excluded as it had FPKM values of 4.7 and 4.0 in the PB and BMA samples respectively, but the value in the BMT sample was 623.7, i.e. >130 fold higher. Following these exclusions, evaluable data was obtained from 10,363 genes, representing 51.6% of genes covered by the RNA-seq system employed. Next, in an attempt to further reduce the potential for influence of stromal tissue, the proposed gene function and expression profiles were examined using the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/gene), and genes with a predominantly stromal expression profile or function were excluded.

Figure 3.9 Assessment of RNA quality by Agilent Bioanalyser 2100. Bands corresponding to 28S and 18S ribosomal RNA running at 5kb and 1.9 kb respectively are clearly visible in all samples. kb = kilobases.



There were 472 non-stromal genes expressed at ≥2-fold higher level in the BMT than the PB sample, of which 146 were related to the cell-cycle. These included members of the MCM family, e.g. MCM4, MCM6, MCM8, and MCM10, *GMNN* encoding geminin, and *MKI67* encoding Ki-67, and targets of drug development such as *CDC7* and the polo-like kinases (Table 3.4). In contrast, a key regulator of the cell cycle, *CDKN1C*, was found to be expressed at >2-fold higher level in the PB than the BMT sample. When expression of these cell-cycle genes was viewed in aggregate, the profile of the BMA sample was clearly more akin to that of the PB than the BMT sample (Figure 3.10). Furthermore, differences in expression between the samples were not restricted to the cell cycle. For example, 14 different transcription factors apparently unrelated to the cell cycle were down-regulated in the BMA/PB samples as compared to the BMT sample (Table 3.5). These transcription factors have putative roles that include AP-1 signalling and the stress response. Interestingly, in a previous study that looked at differences between CD34+ myeloblasts derived from BMA or PB samples, *FOSB* and *JUN* were expressed at higher levels in marrow blasts compared to PB in all 5 paired samples studied (Cheung *et al*, 2009) (Table 3.6)

3.3.3 Impact of source of blasts for published gene expression studies

In order to assess whether this haemodilution with PB-derived G_1-arrested blasts may have an impact on other biological parameters, it was decided to see whether these changes would also been seen in studies of gene expression profiling. In 2004 Bullinger and colleagues published a study using cDNA microarrays to determine the levels of gene expression in peripheral blood or bone marrow aspirate samples from 116 adults with AML (Bullinger *et al*, 2004). Using unsupervised hierarchical clustering, they identified molecular subgroups with distinct gene expression profiles and identified a signature of 133 genes that were used to define prognostic subclasses and predict survival in an independent validation group. Follow-up studies verified the utility of this gene set, again using blood and aspirate samples (Radmacher *et al*, 2006). This signature was therefore assessed using the RNAseq data from the BMT, BMA, and PB samples. The resulting heatmap shows that, although there are clear similarities between the blast cells derived from the different sources, the BMA and

Table 3.4 Cell cycle-related genes down-regulated in blast cells from the PB sample compared to the BMT sample. Genes mentioned in Chapter 1 in the context of the G_1/S phase transition are in bold.

Removed for Copyright

Removed for Copyright

Removed for Copyright

Removed for Copyright

Removed for Copyright

Figure 3.10 RNA expression of 146 cell cycle-related genes that had ≥2 fold difference in expression between the BMT and PB samples. The colour range refers to the median normalised $\log_e 2$ values across samples.

Removed for copyright

Table 3.5 Fold differences in expression of transcription factors without a clear cell-cycle role that were down-regulated in the PB compared to the BMT sample. Functions were determined from the NCBI database.

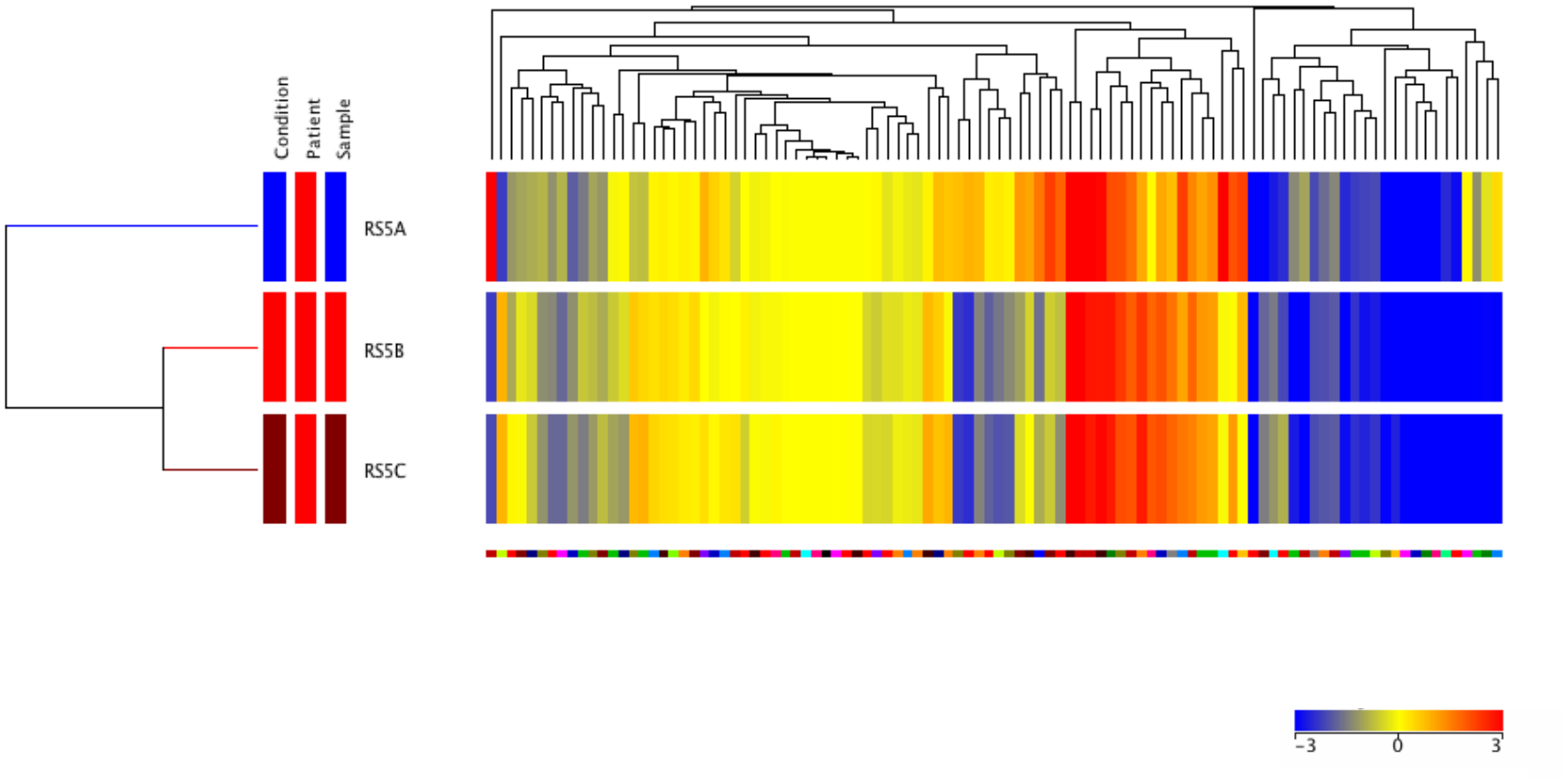
Removed for Copyright

Table 3.6 Fold difference in expression between paired samples from the patient studied of differentially expressed genes identified in the study by Cheung *et al* (2009).

Gene	Function	BMT vs BMA	BMT vs PB	BMA vs PB
RASGEF1B	G-protein signalling	1.61	2.41	1.49
MCL1	Enhances cell survival by inhibiting apoptosis, while an alternatively sliced isoform promotes cell death	1.14	2.34	2.05
DUSP1	MAP kinase phosphatase	1.65	5.63	3.42
ATF3	Transcription factor involved in stress response	2.25	30.38	13.49
FOSB	AP-1 transcription factors	6.35	14.22	2.24
RGS1	G-protein signalling	12.69	33.17	2.61
KLF6	Kruppel-like transcription factor	0.66	3.47	5.26
KLF4	Kruppel-like transcription factor	2.81	6.45	2.29
RGCC (C13orf15)	Regulates cell cycle progression	1.51	2.58	1.71

PB samples still clustered separately from the BMT sample (Figure 3.11). Indeed, there were a number of genes for which the expression profiles between the BMT and the BMA/PB samples were distinctly different. There were 45 genes with a significant discrepancy between the BMT and the BMA/PB samples (>2-fold difference or with FPKM >5 for one tissue but absent from the others) (Table 3.7). This represents 34% of all genes in the signature. Of note, there were multiple genes with an apparently "stromal" role that formed part of this signature, including the previously mentioned *COL1A1,* as well as *PMP22*, which is related to myelin, and *FBLN2*, which is related to the extracellular matrix.

Figure 3.11 Gene expression in the BMT, BMA, and PB samples of the 133 genes in the prognostic-predictor signature defined by Bullinger *et al.* (2004). The colour range refers to the median normalised $\log_e 2$ values across samples.



3.4 Discussion

There are several reasons why the investigation of the cell cycle status of leukaemia cells from patients with AML is potentially important. As discussed in chapter 1, excess proliferation is a common feature of AML and there are well-established routes to dysregulation. Studies of the cell cycle may therefore inform on fundamental biological aspects of the disease. Furthermore, the main treatment regimen used to treat patients with AML contains two agents (daunorubicin and Ara-C) that exert their effect, at least in part, in a cell cycle-dependent way. In addition, many of the newer agents in early phase trials target key cycle proteins. Defining the cell cycle characteristics of disease may therefore inform as to why patients respond differently to current and future therapies. However, despite the large number of studies trying to link the cell cycle status to clinical outcome, results have been inconsistent and even contradictory. A possibility is that these discrepancies relate to the different techniques used for analysis and, perhaps more fundamentally, to the source of blast cells. This chapter investigated differences in cell cycle parameters according to the source of blast cells and assessed how these differences might impact on the results of biological and clinical studies.

Although previous studies examining the relationship between cell cycle status or kinetics of cells obtained from patients with AML have consistently shown lower values for the proportion of cells in S phase or $S/G_2/M$ phase in PB than BMA samples, and for BMA than BMT samples, few studies have taken paired samples to show that in an individual patient the results from cells obtained from BMAs and from BMTs are discordant, and none have also looked at PB samples as well such that cell cycle status in all three 'compartments' may be compared. This question is fundamental to measuring the cell cycle in AML as BMA samples are variably haemodilute. This may in theory introduce artifact and an inaccurate representation of the activity in the marrow.

The studies presented here used immunohistochemistry and immunocytochemistry to compare expression of cell cycle-related proteins in BMT, BMA and PB samples from eight patients with AML. This allowed direct comparison of results obtained from blasts from the different tissue compartments and the degree of variability and artifact introduced depending on the source of cells. Although a similar number of blasts from

BMT and PB samples were in-cycle as defined by MCM2 expression, other parameters measured were significantly different between the two. A median of 63% of the blasts from BMT samples were actively proliferating as defined by expression of MIB-1, and 24% had progressed beyond G_1 into $S/G_2/M$ as defined by expression of geminin. The respective figures for MIB-1 and geminin from PB cells were 4% and 1%, indicating that the majority of blasts in PB exist in a G_1 arrested state and are not actively proliferating.

Haemodilution of BMAs with these out-of-cycle PB-derived blasts means that cell cycle analysis using BMA samples are not accurately representing the true *in vivo* cell cycle parameters and may give an artificially low result for the number of cells actively cycling, and the numbers of cells that have progressed beyond G_1. A key point is that although the geminin/MIB-1 ratio in an individual patient can be similar in the BMT and BMA samples, in some patients the levels are markedly different. For example, patient 4 had a presenting WCC of 2 $x10^9$/l and geminin/MIB-1 ratios of 0.08 and 0.07 in the BMT and BMA samples respectively, indicating that the speed of cycling measured from these compartments was essentially the same. In contrast, in patient 8, who had a presenting WCC of 365 $x10^9$/l, the geminin/MIB-1 ratios were 0.59 and 0.15 respectively, indicating that the speed of cycling determined when using cells from the BMA sample was almost 4-fold lower than the true value as measured using the BMT sample. It is also noteworthy that the values for geminin/MIB-1 ratios in the PB blasts were not correlated with those in BMT samples, and in some patients were markedly higher in the PB. For example, for patient 4, the geminin/MIB-1 ratio in the PB sample was 0.5, far higher than the levels of 0.09 and 0.07 found in the BMT and BMA samples. It is likely that this reflects the fact that only 2% of blasts in the PB were MIB-1 positive, with evaluation of so few events leading to a wide margin of error for the geminin results and subsequently the geminin/MIB-1 ratio.

In work performed in conjunction with these studies (Sellar *et al*, 2016) it was predicted that, because of the larger number of cells in a given volume of PB, the impact of haemodilution and artificially low measured geminin/MIB-1 ratios, would be greatest in those patients with a higher WCC. Therefore, if using BMA samples, the measured proliferative drive may be paradoxically lower in those patients with the highest proliferative drive. In the cohort of patients studied in this thesis, patient 8 had the

highest WCC and also the second highest geminin/MIB-1 ratio in their BMT sample. However, they had the second lowest geminin/MIB-1 ratio in their BMA sample. This further highlights the inaccurate and discrepant results obtained when using BMAs.

Importantly, the results of the quantitative analysis of $S/G_2/M$ cells as determined by IHC/ICC for geminin were highly correlated with results obtained by flow cytometry following staining with propidium iodide. This demonstrates that the results obtained from the cell cycle analysis are reproducible when different techniques are used. This correlation was seen in cells obtained from BMTs and not just BMAs and PB. Furthermore, as correlation does not necessarily imply equivalence, it is noteworthy that the results were also shown to be essentially equivalent by Bland-Altman analysis, a statistical technique that better assesses whether different methods of measurement give the same result. The cell cycle flow cytometry assay was designed to assess the overall percentage of cells in $S/G_2/M$. This is the same population of cells that should be positive for geminin. However, it would be of interest to perform a more extensive analysis by assessing $S/G_2/M$ after gating on MIB-1 positive cells. This would allow comparison of the two techniques not only for the assessment of actively cycling cells but also for the proportion of actively cycling cells that have progressed beyond G_1 as a surrogate for the speed of cell cycling.

In aggregate, these results show that using leukaemic cells from BMAs does not give an accurate reflection of the cell cycle status of cells in the bone marrow. Studies that use such results, either in whole or as part of an equation, to define cell cycle status are therefore likely to be inaccurate. As such, variations between patients will depend not just on true differences in cell cycle status, but also on the level of haemodilution as dictated by WCC, aspirate volume, and quality of the samples. These data thus demonstrate that for accurate cell cycle analysis of cells from patients with AML, biopsies must be used. The studies also demonstrate that IHC/ICC can give equivalent results to flow cytometry, with the added advantage that is more suited to the use of archived tissue.

This haemodilution of BMA samples with PB-derived G_1 arrested blasts is likely to have an impact on other biological parameters. For example, gene expression profiling has been extensively studied in AML (Shivarov & Bullinger, 2014), but such studies

use PB and BMA samples to derive the results. To examine whether sample source is important, and to enable comparative analysis of cells with equivalent blast populations without the need for processing that could potentially alter gene expression, samples were obtained from a patient who had >90% blasts in their BMT, BMA, and PB. For each source of cells, RNA expression profiles were obtained from RNAseq data. Analysis was focused on "non-stromal genes" with FPKM values ≥20 in the PB and BMT samples and ≥2-fold difference between the PB and BMT values. Despite being a near pure blast population from the same patient, there were significant differences in profiles between the BMT and PB samples in expression of key cell cycle genes, including in genes that had already been demonstrated to be differentially expressed at the protein level by IHC/ICC. Furthermore, cluster analysis showed that the expression profiles for cell cycle-related genes was most similar between the BMA and PB samples. In addition, there were a number of genes that are not normally regarded as "cell-cycle" genes that were also differentially expressed, including key transcription factors. Again, the BMA and PB samples were more similar to each other than to the BMT sample. Although it is not possible to say that the cell populations were identical, small differences in cellular composition are unlikely to swamp the cell cycle signal of the predominant blast populations and account for the large differences seen between the samples.

Differences in the "non cell cycle" gene groups may reflect true differences in expression between the different blast populations or, potentially, differences in the relative abundance of other cell types. For example, some lymphocyte genes were expressed at higher levels in PB than in the BMA or BMT samples (e.g. for CD3D, which is in the signature gene list derived by Bullinger *et al* (2004), the FPKM values were respectively 5.5, 4.1, and 1.6). This was one of the reasons for restricting the main analysis to the more highly expressed genes (FPKM ≥20 in both BMT and PB) on the basis that this should limit the impact of low abundance cells that nonetheless may have potentially large fold changes in their frequency. Furthermore, there were multiple genes with crucial roles in myeloid cell biology that were differentially expressed between the compartments (Table 3.5) that have been implicated as having biological or prognostic importance in AML. These include *FOSB* and *JUN,* which supports the finding of a previous study showing that these transcription factors had a higher expression in cells from BMAs than PB. *JUN* has recently been shown to be a key

regulator of the unfolded protein response in AML (Zhou *et al*, 2016).

To further explore the impact of these differences on gene expression profiling, the FPKM values for this same patient were examined in the context of the signature of 133 genes found to be predictive for clinical outcome by Bullinger *et al* (2004). Once again, the results from cells obtained from the BMA and PB were more closely aligned than the results from the BMT cells. When the gene list was examined for those genes that were most differentially expressed (Table 3.7) many of the genes with the biggest demonstrable differences could be seen to be to of questionable direct relevance to myeloid cells. Indeed, many were more related to lymphocyte biology e.g. *IL6ST* and *CD3D*, or stroma, e.g.*COL1A1*.

A concern, therefore, is whether the predictive effects of this gene set, or of studies of individual genes, predominantly reflects differences in the samples used and the level of marrow contamination with peripheral blood blasts or lymphocytes. For some studies, cells from BMAs and PB have been used in the same set and used interchangeably. In fact, studies showing equivalence may be correct, but simply because, once the diagnostic BMA sample has been used for morphology, flow cytometry, cytogenetic, molecular analysis, and clinical trials, the remaining sample that is available for biobanking and subsequent expression profiling is so haemodilute that it is essentially PB-derived cells.

Although superior in accuracy for cell cycle analysis, and presumably for cell cycle profile by gene expression profiling, there are several caveats before unequivocal advocacy of BMT samples for the latter analysis can be made. Firstly, although the results were clearly different between the BMT and BMA/PB cells in the patient studied here, they do represent only one patient. Further patients would need to be assessed to see whether samples cluster by patient, by source of cells, or by a combination of both these factors. However, a potential problem in assessing further patients is the relative ease of obtaining cells from BMTs and in sufficient numbers for reliable analysis. Obtaining relatively pure populations of cells is possible but would limit the number of cells, and it is not clear how such processing would affect RNA stability prior to RNA extraction and analysis. Nevertheless, future comparisons of paired samples would require equivalent numbers of blast cells from the different tissue sources in order to

minimise the impact of different proportions of myeloid cells on the analysis.

A further consideration is the potential impact of these cell cycle analyses for clinical studies. As described in the introduction, there are a number of agents that target cell cycle proteins that are in advanced stages of clinical trials. It is notable that two of these targets, PLK1 and Aurora kinase B, were amongst the genes with the most discrepant RNA expression levels (Table 3.4). This could mean that attempts to correlate expression with clinical response would be compromised. The same caveat exists for PIM1 and the inhibitors of PI3K. Both of these genes were differentially expressed between BMT and PB blasts, and these are important pathways in the development of novel therapeutics to treat AML.

Finally, these studies suggest that circulating blasts will be less susceptible to treatment with cell-cycle phase specific agents such as Ara-C, the mainstay of current AML therapy. Nevertheless, by virtue of not having entered quiescence, these cells may be capable of rapid proliferation if they recirculate back to the BM. This is particularly relevant to the potential use of CXCR4 inhibitors to mobilise blast cells into the circulation prior to the administration of chemotherapy (Nervi *et al*, 2009).

In summary, the data from this chapter demonstrate that the blasts from PB of AML patients exist in a G_1-arrested state and, as a result of haemodilution, this leads to inaccurate information of the cell cycle status of bone marrow blasts as assessed by experiments performed on BMA samples. For accurate assessment of cell cycle state cells from BMTs must be used. These data have potential implications for biological studies of gene expression profiling, the design and assessment of efficacy of drug trials, the design of new therapies, and clinical studies of prognosis and risk stratification.

CHAPTER 4. DEFINING A COHORT OF PATIENTS WITH AML

4.1 Introduction

In Chapter 1 the main diagnostic and prognostic criteria for patients with AML were discussed, and these have contributed to the establishment of well-defined patient cohorts. Such cohorts are crucial to allow accurate comparisons of treatment approaches and new prognostic variables within trials and between trials to aid clinicians and patients. In addition to establishing cohorts in the context of clinical trials, 'real-world' patient cohorts may be established from population registries (Derolf *et al*, 2009). Cohort comparisons have consistently shown that the outcomes of patients under 60 years of age have improved over time, whilst patients over 60 have seen little benefit. Furthermore, with the exception of patients with APL in whom the treatment approach has changed considerably, improvements have been modest and are an "aggregation of marginal gains", with the greatest impact potentially seen from improved supportive care and transplant outcomes rather than novel treatment agents.

The corollary of such well-defined cohorts with robust outcome data is that any study that seeks to make accurate statements about new treatments or new prognostic markers must use or establish a cohort that demonstrates demographic and outcome equivalence to published cohorts. In Chapter 3 it was shown that for accurate cell cycle analysis in patients with AML, biopsy samples must be used. The focus of the work presented in this chapter was to establish a cohort of patients with AML who had biopsies available for IHC analysis of DNA replication licensing proteins and cell cycle status. In order to allow interpretation of cell cycle dynamics in a useful clinical and biological context, the analysis set out to examine general equivalence of this cohort with large published cohorts in terms of the presenting genetic features, the robustness of prognostic factors, the response of patients to treatment, and overall patient outcomes.

4.1.1 Required features of the cohort

In chapter 1 the enduring importance of clinical features such as age, presenting WCC,

and cytogenetics in relation to clinical outcome was emphasised. For patients within the intermediate cytogenetic risk group, the mutation status of *FLT3* and *NPM1* are also of prognostic significance and need to be defined.

4.1.2 FLT3

FLT3 (Fms-like tyrosine kinase 3), also known as foetal liver kinase-2 (FLK-2) or stem cell kinase-1, is a 993 amino acid class III membrane-bound receptor tyrosine kinase encoded by a 24 exon gene located on chromosome 13 (13q12). FLT3 is expressed on the surface of normal HPCs within the BM, thymus and liver, as well as on the surface of more mature blood cells including monocytes, and has a crucial role in normal haematopoiesis. It is also expressed on the leukaemic cells from the majority of patients with AML and acute lymphoblastic leukaemia (ALL), and the blast crisis phase of chronic myeloid leukaemia (CML) (Stirewalt & Radich, 2003).

4.1.2.1 Mutations in the juxtamembrane domain of *FLT3*

In 1996, using RT-PCR, Nakao and colleagues identified unexpectedly long *FLT3* transcripts that were shown to result from a region of duplicated genomic DNA within the juxtamembrane (JM) domain encoded by exons 14 and 15, and called them *FLT3* internal tandem duplications ($FLT3^{ITD}$) (Nakao *et al*, 1996). Subsequently, these duplications have been shown to be somatic, to range from 3 up to approximately 400bp and, as they are always multiples of 3bp, to remain in-frame. They disrupt the negative regulatory activity of the JM domain resulting in constitutive activation of the receptor and enhanced RAS, MAPK, and STAT5 signaling (Stirewalt & Radich, 2003). This alters the normal physiological role of FLT3 in cell proliferation, differentiation and survival.

Screening of large cohorts of patients has demonstrated that $FLT3^{ITD}$ occur in approximately one quarter of cases of AML in young adults and up to a third of patients with cytogenetically normal AML (Table 4.1). However, the reported incidence varies

considerably, partly relating to heterogeneity in the age and cytogenetic status of the patients studied. In up to 23% of *FLT3*^{ITD} patients more than one *FLT3*^{ITD} may be present, and in some patients up to 5 have been reported (Kottaridis *et al*, 2003).

4.1.2.2 Mutations in the tyrosine kinase domain of *FLT3*

In up to 7% of de novo AML, missense point mutations have been detected leading to activating mutations affecting codons D835 and I836 in the second tyrosine kinase domain (TKD) (Abu-Duhier *et al*, 2001; Yamamoto *et al*, 2001). These mutations are thought to disrupt key amino acids that stabilise the activation loop in a closed configuration (Griffith *et al*, 2004). Although the exact significance of TKD point mutations has proved controversial, they appear to differ from *FLT3*^{ITD} in that they are less potent in their transforming ability, less susceptible to tyrosine kinase inhibition with drugs, and have less clearly defined prognostic implications (Mead *et al*, 2008a; Mead *et al*, 2008b).

4.1.2.3 Biological consequences of *FLT3*^{ITD}

FLT3^{ITD} promotes ligand-independent dimerisation, auto-phosphorylation, and constitutive activation of the receptor, which promotes ligand-independent proliferation and, in model systems, a block of myeloid differentiation. In a mouse bone-marrow transplant model, the introduction of *Flt3*^{ITD} induced a myeloproliferative phenotype without demonstrable leukaemia (Kelly *et al*, 2002). In a transgenic model, *Flt3*^{ITD} under the control of a *Vav* promoter also resulted in a myeloproliferative disorder with long disease latency, but in some cases in B or T-lymphoid disease, again without a clear acute leukaemia phenotype (Lee *et al*, 2005). Nevertheless, the transforming potential of *Flt3*^{ITD} is clear when combined with other genetic lesions. In mouse models, genetic lesions that result in

Table 4.1 The major studies published on the impact of a *FLT3*^{ITD} mutation on CR and OS rates for patients with AML.

No. of patients	Median Age, yrs (range)	*FLT3*^{ITD} (% of patients)	CR	OS	Reference
201	49 (15-85)	23	NS	Reduced	(Kiyoi *et al*, 1999)
106	Mean 41 (15-74)	13	NR	Reduced	(Abu-Duhier *et al*, 2000)
854	41 (16-63)	27	NS	Reduced	(Kottaridis *et al*, 2001)
140	67 (56-88)	29	NS	NS	(Stirewalt *et al*, 2001)
224	41 (29)	30	NS	Reduced	(Frohling *et al*, 2002)
979	NR	20	NS	NS	(Thiede *et al*, 2002)
563	NR	22	NS	NS	(Schnittger *et al*, 2002)
159	44 (15-65)	25	NS	NS	(Boissel *et al*, 2002)
146	NR	19	NS	Reduced	(Moreno *et al*, 2003)
234	NR (children)	12	Reduced	Reduced	(Zwaan *et al*, 2003)
109	70 (60-90)	18	NS	NS	(Andersson *et al*, 2004)
630	NR (0-21)	12	NS	NS	(Meshinchi *et al*, 2006)

Abbreviations: CR, complete remission; NR, not reported; NS, not significant; OS, overall survival

more rapid transformation when combined with a *Flt3*^{ITD} include PML-RARα (Sohal *et al*, 2003), NUP98-Hox (Palmqvist *et al*, 2006), AML1-ETO (Schessl *et al*, 2005), and MLL-ENL (Ono *et al*, 2005).

Further evidence that *FLT3*^{ITD} are often cooperating events in disease transformation or evolution rather than initiators of leukaemia comes from clinical studies. In many patients the mutant fraction is <25% at disease presentation. This suggests that fewer than half of the leukaemic cells in these patients are heterozygous for the mutation and, therefore, implies that the mutation is not present in the majority of the leukaemic cells, although an alternative explanation for lower mutant allele burden in some cases is the presence of contaminating non-leukaemic cells within the sample. Furthermore, *FLT3*^{ITD} can be highly unstable and at relapse may be lost in up to 20% of patients, suggesting persistence of an earlier leukaemic clone lacking the *FLT*^{ITD} (Kottaridis *et al*, 2002).

4.1.2.4 Detection of *FLT3*^{ITD}

Detection of *FLT3*^{ITD} can be performed accurately, rapidly, and with minimal expense. PCR products can be generated using primers flanking exons 14 and 15 in which the majority of *FLT3*^{ITD} occur, and the wild-type (*FLT3*^{WT}) band and any larger band representing a mutant readily separated using agarose gel electrophoresis (Figure 4.1). However, as will be discussed in the later section on clinical outcome, the mutational burden (ratio of *FLT3*^{ITD} to *FLT3*^{WT} alleles or percentage of *FLT3*^{ITD}) may be important to both clinical features and clinical outcome, and gel electrophoresis does not accurately provide this information. Instead, using a fluorescently-labelled primer, the labelled PCR products can be separated according to size using a capillary system fragment analyser and quantified, and hence the mutational burden relative to the smaller WT fragment can be established (Figure 4.2).

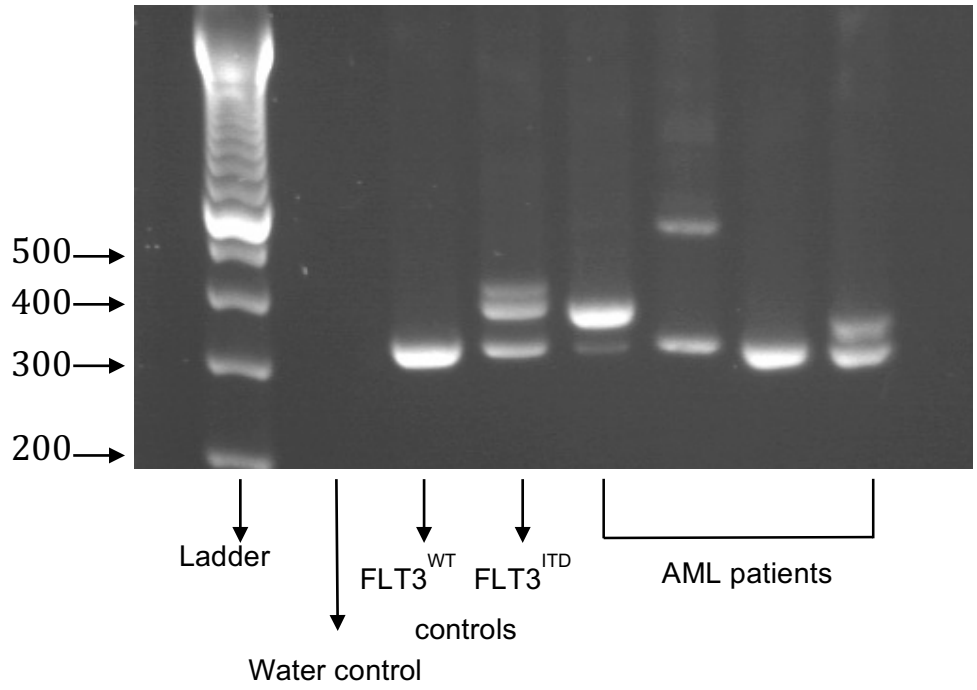


Figure 4.1 Analysis of *FLT3* mutation status by PCR and agarose gel electrophoresis. Mutations (ITD) are seen as larger slower moving bands relative to the wild type (WT).

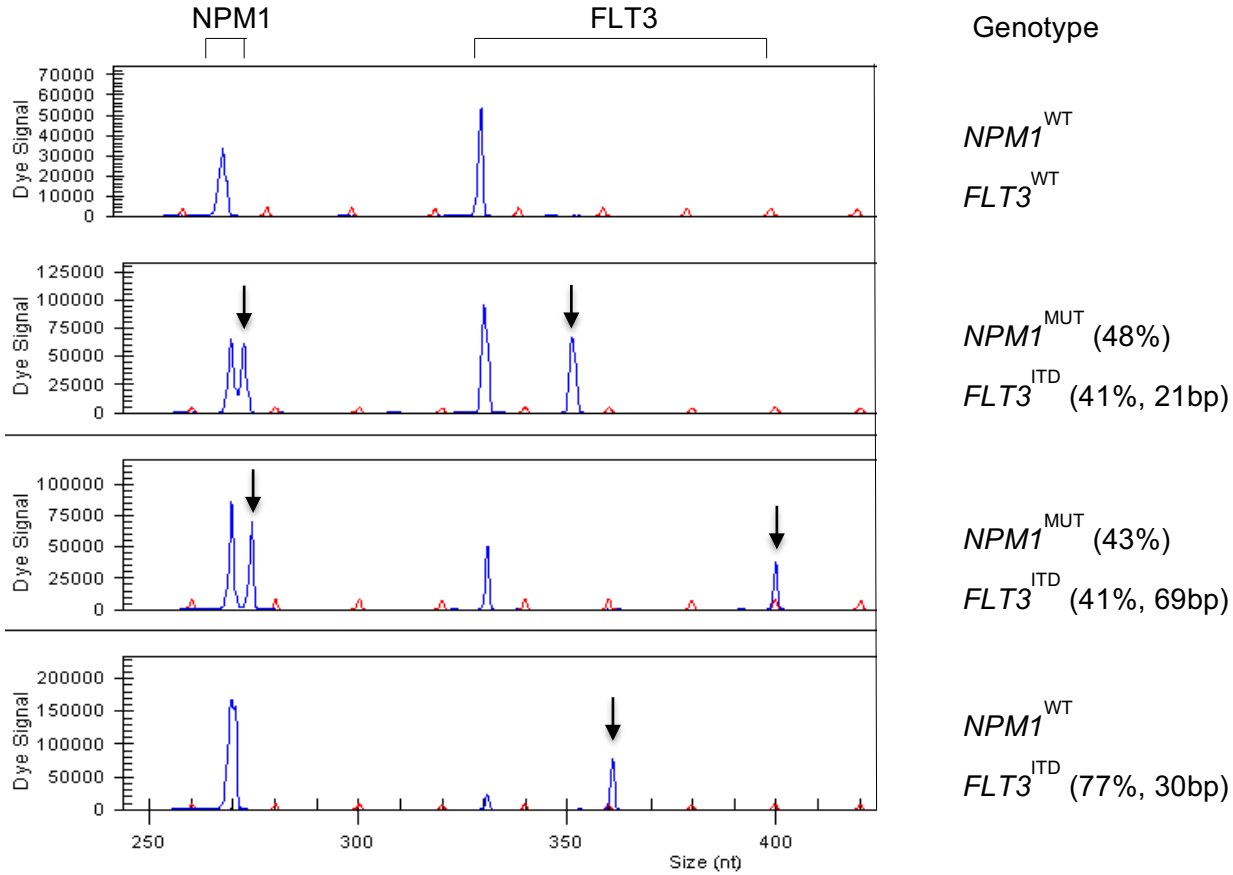


Figure 4.2 Analysis of *FLT3* and *NPM1* mutation status and mutation burden using fragment analysis.

The larger fragments for the respective genes representing the mutant allele are indicated by an arrow.

4.1.2.5 Clinical characteristics of AML patients who have *FLT3*^{ITD}

Studies of the clinical features of patients with *FLT3*^{ITD} have been generally consistent in showing an association with a high presenting WCC and higher percentage of blasts in marrow samples (Kottaridis *et al*, 2001). Studies of paediatric cohorts have generally shown a lower incidence of *FLT3*^{ITD} than those seen in adult patients (Meshinchi *et al*, 2006) (Table 4.1).

Correlation with FAB subtypes has demonstrated that *FLT3*^{ITD} are particularly common in patients with M3 (APL), more specifically the microgranular variant (M3v), as well as AMLs with a monocytic/monoblastic component (M4 and M5a). Some, but not all studies, have observed an increased frequency in patients with *de novo* AML compared to those with secondary AML arising as a transformation from myelodysplasia. In a recent study using strictly defined disease ontogeny, there was a trend towards an increased presence of *FLT3*^{ITD} in de novo AML over secondary AML, although this was not statistically significant (Lindsley *et al*, 2015). In terms of cytogenetic risk groups, in addition to being found more commonly in patients with t(15;17), *FLT3*^{ITD} are also more common in patients with t(6;9). With these notable exceptions, they are otherwise found at reduced frequency in patients with favourable and adverse cytogenetics, being significantly enriched in the intermediate risk cytogenetic risk group.

4.1.2.6 Clinical outcome of patients with *FLT3*^{ITD}AML

Details of the major studies investigating the prognostic importance of *FLT3*^{ITD} mutations in >100 non-APL AML patients are summarised in Table 4.1. Except in patients with APL, *FLT3*^{ITD} are generally associated with poor prognosis and decreased OS (Kiyoi *et al*, 1999; Kottaridis *et al*, 2001; Frohling *et al*, 2002; Small, 2006), although not all studies have shown this (Stirewalt *et al*, 2001; Boissel *et al*, 2002; Schnittger *et al*, 2002; Thiede *et al*, 2002). Such discrepancies may relate to differences in ages between cohorts, treatment approach including rates of allogeneic transplant, mutation frequency and the relative mutant burden. Most studies have not shown

decreased CR rates but rather that inferior survival comes from the significantly increased CIR.

In one study size (length) of *FLT3*^{ITD} influenced survival, with longer *FLT3*^{ITD} conferring a worse prognosis, although this has not been found in subsequent studies (Ponziani *et al*, 2006; Stirewalt *et al*, 2006; Gale *et al*, 2008). In addition to the low mutant levels (<25% of total *FLT3* alleles) mentioned earlier, some patients have a mutant level greater than 50%, suggesting loss of the WT *FLT3* allele. This occurs by uniparental disomy and is associated with a particularly poor outcome (Gale *et al*, 2008). In the study by Thiede and colleagues, although the OS was non-significant for *FLT3*^{ITD} overall, for patients with higher mutant burden (greater than the median mutant:WT ratio of 0.78), there was a demonstrably inferior OS (Thiede *et al*, 2002).

However, a potentially key confounding factor is that many of the earlier studies pre-dated the discovery of mutations in the *NPM1* gene (*NPM1*^{MUT}), a mutation that is both positively correlated with *FLT3*^{ITD} and has significant prognostic implications.

4.1.3 Nucleophosmin

Nucleophosmin (NPM1), encoded by the *NPM1* gene localised at chromosome 5q35, is a nucleocytoplasmic shuttling protein that is normally most prominent in nucleoli. It has diverse proposed functions including acting as a molecular chaperone, a target of CDK2-cyclin E complexes in centrosome duplication, and involvement in the regulation of the p14^{ARF}–p53 tumour suppressor pathway crucial to DNA repair and genomic stability (Grisendi *et al*, 2006). Overexpression of NPM1 has been found in several types of solid tumours, and the level of overexpression has been linked to stage, disease recurrence and progression (Grisendi *et al*, 2006).

4.1.3.1 Mutations in *NPM1*

In 2005, Falini and colleagues described abnormal cytoplasmic location of NPM1 in approximately a third of a cohort of patients with AML, with an even higher incidence

in patients with cytogentically normal AML (CN-AML) (Falini *et al*, 2005). In these patients they identified a number of mutations within exon 12 of the *NPM1* gene, most commonly 4 bp insertions, with the most frequent of these (type A mutations) a duplication of the TCTG tetranucleotide at positions 956 through to 959. Other mutations (B-D) were also 4 bp insertions but of different nucleotide sequence. A further 2 mutations with a larger 9bp insertion (E and F) were also described. Regardless of the exact nature of the mutation, the consequence was to cause a frameshift in the C-terminal region of the protein. All six mutant proteins had alteration in at least one of the tryptophan residues at positions 288 and 290 and shared the same terminal amino acid sequence (VSLRK). Transfection experiments showed that the presence of this abnormal sequence correlated with abnormal protein localisation. In addition to leading to the loss of a nuclear localisation signal, Mariano and colleagues have subsequently shown that the new sequence of amino acids is a functional nuclear export signal (Mariano *et al*, 2006).

Since this original description, almost 50 *NPM1* mutation variants have been described, which are almost exclusively within exon 12. Further studies on larger cohorts of patients have consistently shown *NPM1* mutations in approximately one third of patients, with rates of over 50% in NK AML, and with the overwhelming majority of mutations being of Type A. Intriguingly, mutation rates in childhood cohorts are consistently <10% and predominantly non-type A mutations (Cazzaniga *et al*, 2005; Brown *et al*, 2007; Hollink *et al*, 2009).

4.1.3.2 Biological consequences of *NPM1* mutations

There are a number of proposed mechanisms by which the abnormal localisation of the mutated NPM1 protein may contribute to leukaemogenesis. These include a gain of function, with the relocated mutant NPM1 protein having increased interactions with cytoplasmic proteins and potential new interaction partners, or a loss of function as the result of absence from or decreased abundance in the nucleolus. WT NPM1 is also affected through the formation of heterodimers, with loss of WT protein to the cytoplasm. Experimental evidence of specific effects includes loss of activity of ARF and activation of c-MYC (den Besten *et al*, 2005; Colombo *et al*, 2006; Bonetti *et al*,

2008). Further *in vitro* studies have shown that mutant NPM1 can promote oncogenic transformation of primary cells in cooperation with E1A (Cheng *et al*, 2007).

However, many of the insights into pathogenesis have come from animal models, and these support the suggestions that the disease-causing potential of these mutations results from both haploinsufficiency as well as a specific role for the mutated cytoplasmic form of NPM1. *Npm1* knockout mice show increased levels of genomic instability and susceptibility to cancer, and haploinsufficient (*Npm1* mutant/null) mice develop spontaneous tumours with a preponderance of myeloid malignancies (Sportoletti *et al*, 2008). Introduction of the mutation results in excess proliferation of myeloid cells in both murine and zebrafish models (Bolli *et al*, 2010; Cheng *et al*, 2010). In the mouse this was a myeloproliferative disorder, in contrast to the zebrafish where expansion of more primitive cells and progenitor cells was seen. Crucially, the introduction of mutant protein was insufficient to induce frank AML. There are numerous explanations for this including inability of sufficient follow–up, non-pathophysiological levels of mutant to wild-type protein, protein expression in a myeloid precursor without the potential for transformation, or simply lack of requisite cooperating events.

4.1.3.3 Detection of *NPM1* mutations

In the original report by Falini *et al* (2005), the presence of mutations was detected by immunohistochemistry (IHC), and confirmed by Sanger sequencing in a subset of patients. Whilst IHC remains a reliable way of detecting mutant NPM1 protein, it has largely been superseded by molecular analysis. For initial screening, fragment analysis can rapidly and reliably detect insertions causing size change in the PCR product (Thiede *et al*, 2006). Such a method does not discriminate type A mutations from rare variants, although there is no clear published evidence that such variants are clinically distinct in terms of prognosis. Other groups have used mutation-specific probes and melting curve analysis for screening, and these analyses are able to discriminate between type A, B, and D mutations (Schnittger *et al*, 2005). Such techniques have proved useful for screening large number of patients, with the potential for multiplexing in order to simultaneously screen for other genetic mutations such as $FLT3^{ITD}$. These

techniques are also relatively sensitive in being able to reliably detect mutations down to mutant levels of approximately 5%. In practice, these levels of sensitivity are not required at diagnosis as *NPM1*^{MUT} are usually heterozygous and found in all leukaemic cells of *NPM1*^{MUT} AML, implying that they are early events in leukaemogenesis.

In addition, *NPM1*^{MUT} are one of the most stable mutations, being rarely lost at relapse, and only very occasionally found in remission. As such, *NPM1* has been pursued as a potentially useful marker for minimal residual disease monitoring. In this scenario, techniques with a sensitivity of 5% are inadequate. Quantitative real time PCR techniques have been developed that may detect one abnormal cell in 100 000. The clinical feasibility and utility of such an approach has recently been demonstrated (Ivey *et al*, 2016).

4.1.3.4 Presenting clinical features of *NPM1*^{MUT} patients

Falini *et al* (2005) described several clinical features of patients with AML containing the newly discovered *NPM1* mutations, including an association with CN-AML, a high frequency of *FLT3*^{ITD}, and the absence of CD34 and CD133 on blasts. Subsequent studies on large numbers of patients have broadly confirmed these findings and made additional observations, as summarised in Table 4.2. These features are deemed to be sufficiently distinct that AML with mutated *NPM1* is a separate entity in the 2016 revision of the WHO classification (Arber *et al*, 2016).

4.1.3.5 Clinical outcome for patients with *NPM1*^{MUT}

A number of studies have investigated the role of *NPM1*^{MUT} in the outcome of patients with AML (Table 4.3). The majority of these studies also take into consideration the impact of *FLT3*^{ITD}. The studies are in general agreement that in younger adult patients, in contrast to *FLT3*^{ITD}, *NPM1*^{MUT} predict for

Table 4.2 Clinical and genetic features of patients with *NPM1*^{MUT} AML.

Presenting Clinical Features	Higher incidence in females (in most but not all studies) High presenting WCC (independent of *FLT3*^{ITD}) Associated with *de novo* AML, rare in sAML Close association with IR and especially CN-AML More often monocytic component (FAB type M4 or M5) Negative for CD34 in >90% of cases
Genetic Features	Mutually exclusive with t(8;21), t(16;16)/inv(16), and t(15;17) Generally mutually exclusive with *CEBPA* double mutants Heterozygous; early event in leukaemogenesis Stable at relapse Unique gene expression profile including increase in *HOX* genes Distinct microRNA profile (e.g. upregulation of *miR-10a* and *miR-10b*)

Table adapted from (Falini *et al*, 2005)

Abbreviations: IR, intermediate risk cytogenetics; CN, cytogenetically normal; sAML, secondary AML; WCC, presenting white cell count.

Table 4.3 Major studies published exploring the impact of *NPM1* mutations on the outcome of patients with AML.

No. of patients	Disease type/ Treatment	Median Age, yrs (range)	*NPM1*^{MUT}	*NPM1*/*FLT3* genotype	Main outcome findings	Reference
591	*De novo*	Not given (15-60)	35%	Not reported	*NPM1*^{MUT} an independent prognostic factor for better CR	(Falini *et al*, 2005)
300	NK Intensive	Not given (16-60)	48%	20% *NPM1*^{MUT}*FLT3*^{ITD} 29% *NPM1*^{MUT}*FLT3*^{WT} 13% *NPM1*^{WT}*FLT3*^{ITD} 39% *NPM1*^{WT}*FLT3*^{WT}	*NPM1*^{MUT}*FLT3*^{WT} genotype prognostic for improved CR, RFS, OS No difference between other genotypes No benefit for HSCT in *NPM1*^{MUT}*FLT3*^{WT}	(Dohner *et al*, 2005)
275	*De novo* Intensive	44 (15-78)	35%	17% *NPM1*^{MUT}*FLT3*^{ITD}	*NPM1*^{MUT} improved OS, EFS, and DFS independent of CGN, age and *FLT3*^{ITD}	(Verhaak *et al*, 2005)
401	NK Intensive	60 (17-82)	53%	21% *NPM1*^{MUT}*FLT3*^{ITD} 31% *NPM1*^{MUT}*FLT3*^{WT} 11% *NPM1*^{WT}*FLT3*^{ITD} 34% *NPM1*^{WT}*FLT3*^{WT}	*NPM1*^{MUT} improved CR and EFS; trend for improved OS. Favourable impact lost with *FLT3*^{ITD} Better EFS, RFS and OS in *NPM1*^{MUT}*FLT3*^{WT} No difference in outcome between *NPM1*^{WT}*FLT3*^{ITD} and *NPM1*^{MUT}*FLT3*^{ITD}	(Schnittger *et al*, 2005)
107	Intensive	0-18 (35% <5 years)	7% (27% of NK)	1% *NPM1*^{MUT}*FLT3*^{ITD} 6% *NPM1*^{MUT}*FLT3*^{WT} 9% *NPM1*^{WT}*FLT3*^{ITD} 84% *NPM1*^{WT}*FLT3*^{WT}	All NPM1^{MUT} patients achieved CR No deaths from disease at last follow-up, but short follow-up	(Cazzaniga *et al*, 2005)
257	All subtypes Intensive	(15-85)	25%	14% *NPM1*^{MUT}*FLT3*^{ITD} 11% *NPM1*^{MUT}*FLT3*^{WT} 9% *NPM1*^{WT}*FLT3*^{ITD} 66% *NPM1*^{WT}*FLT3*^{WT}	Impaired CR in *NPM1*^{MUT}; no impact of *FLT3*^{ITD} *NPM1*^{MUT} associated with increased RR, no impact on OS Favourable impact of *NPM1*^{MUT} in *FLT3*^{ITD}	(Suzuki *et al*, 2005)
106	NK Intensive	44 (15-65)	47%	18% *NPM1*^{MUT}*FLT3*^{ITD} 29% *NPM1*^{MUT}*FLT3*^{WT} 13% *NPM1*^{WT}*FLT3*^{ITD} 40% *NPM1*^{WT}*FLT3*^{WT}	CR not improved in *NPM1*^{MUT} No difference in outcome for *NPM1*^{MUT} vs *NPM1*^{WT} *FLT3*^{ITD} not prognostic for OS	(Boissel *et al*, 2005)
1485	*De novo* (82%) Intensive	15-87	27% (46% of NK)	11% *NPM1*^{MUT}*FLT3*^{ITD} 16% *NPM1*^{MUT}*FLT3*^{WT} 10% *NPM1*^{WT}*FLT3*^{ITD} 63% *NPM1*^{WT}*FLT3*^{WT}	Improved CR in *NPM1*^{MUT} In multivariate analysis, *NPM1*^{MUT} improved OS	(Thiede *et al*, 2006)

Table 4.3 (continued)						
295	Intensive	9.5 (0-19.5)	8% (20% of NK)	3% $NPM1^{MUT}FLT3^{ITD}$ 5% $NPM1^{MUT}FLT3^{WT}$ 16% $NPM1^{WT}FLT3^{ITD}$ 76% $NPM1^{WT}FLT3^{WT}$	No difference in EFS, or OS for $NPM1^{MUT}$ vs $NPM1^{WT}$ $FLT3^{ITD}$ had greater impact on outcome than $NPM1^{MUT}$, predicting for inferior EFS, and OS. $FLT3^{WT}$ patients trend towards improved EFS in $NPM1^{MUT}$, but no difference in OS.	(Brown *et al*, 2007)
1425	*De novo* Non-APL (92%) Intensive	43	41% (62% of NK)	17% $NPM1^{MUT}FLT3^{ITD}$ 24% $NPM1^{MUT}FLT3^{WT}$ 11% $NPM1^{WT}FLT3^{ITD}$ 47% $NPM1^{WT}FLT3^{WT}$	Improved CR, RR, OS, with $NPM1^{MUT}$ $NPM1^{WT}FLT3^{WT}$ and $NPM1^{MUT}FLT3^{ITD}$ had intermediate prognosis No difference in CR but worse RR, DFS, OS with increasing $FLT3^{ITD}$ mutant level$FLT3^{ITD}$ mutant level was the most significant factor for RR but increased RR even when low level	(Gale *et al*, 2008)
872*	NK Intensive	48 (16-60)	53%	Not reported separately	*not all patients had molecular analysis – n=570 for *NPM1,* n=531 for *FLT3* $NPM1^{MUT}FLT3^{WT}$ associated with increased CR No benefit of HSCT in $NPM1^{MUT}FLT3^{WT}$	(Schlenk *et al*, 2008)
297	*De novo* (96%) Intensive	10	8% (22% of NK)	3% $NPM1^{MUT}FLT3^{ITD}$ 5% $NPM1^{MUT}FLT3^{WT}$ 14% $NPM1^{WT}FLT3^{ITD}$ 78% $NPM1^{WT}FLT3^{WT}$	$NPM1^{MUT}$ improved EFS, not OS $NPM1^{WT}FLT3^{ITD}$ inferior OS $NPM1^{MUT}$ borderline improved EFS and OS in NK	(Hollink *et al*, 2009)
1284	*De novo* Non-APL Intensive	45 (16-59, n=520) 66 (60-85, n=764)	66% 52%	30% $NPM1^{MUT}FLT3^{ITD}$ 37% $NPM1^{MUT}FLT3^{WT}$ 7% $NPM1^{WT}FLT3^{ITD}$ 26% $NPM1^{WT}FLT3^{WT}$ 33% $NPM1^{MUT}FLT3^{ITD}$ 19% $NPM1^{MUT}FLT3^{WT}$ 8% $NPM1^{WT}FLT3^{ITD}$ 40% $NPM1^{WT}FLT3^{WT}$	$NPM1^{MUT}FLT3^{WT}$ increased CR, remission duration, RFS, and OS	(Buchner *et al*, 2009)
148	*De novo* NK Intensive	≥ 60	56%	22% $NPM1^{MUT}FLT3^{ITD}$ 34% $NPM1^{MUT}FLT3^{WT}$ 9% $NPM1^{WT}FLT3^{ITD}$ 35% $NPM1^{WT}FLT3^{WT}$	Overall and for patients ≥70 years, $NPM1^{MUT}$ predicted for increased CR, DFS, and OS. For patients 60-69 years, $NPM1^{MUT}$ had higher CR but not DFS or OS	(Becker *et al*, 2010)
Table 4.3 (continued)						

219	NK	60-83	55%	24% *NPM1*^{MUT}*FLT3*^{ITD} 31% *NPM1*^{MUT}*FLT3*^{WT} 9% *NPM1*^{WT}*FLT3*^{ITD} 36% *NPM1*^{WT}*FLT3*^{WT}	*FLT3*^{ITD} similar CR *FLT3*^{WT} *FLT3*^{ITD} shorter DFS/OS when adjusted for *NPM1* Impact of *FLT3*^{ITD} seen for patients 60-69 but not ≥70 yrs	(Whitman *et al*, 2010)
303	*De Novo* IR	≤60 years	53%	21% *NPM1*^{MUT}*FLT3*^{ITD} 32% *NPM1*^{MUT}*FLT3*^{WT} 10% *NPM1*^{WT}*FLT3*^{ITD} 37% *NPM1*^{WT}*FLT3*^{WT}	In *NPM1*^{MUT}, low *FLT3*^{ITD} burden (ratio <0.5) had similar RR, OS, and DFS to *FLT3*^{WT} *FLT3*^{ITD} ratios ≥0.5 had inferior RR, OS, and DFS	(Pratcorona *et al*, 2013)
1609	Intensive	46	45%	Not reported separately	*NPM1*^{MUT} improved CR and OS; decreased CIR *FLT3*^{ITD} (including low level) increased CIR	(Linch *et al*, 2014)
1312	Intensive Non-intensive	68 75	21%	9% *NPM1*^{MUT}*FLT3*^{ITD} 8% *NPM1*^{MUT}*FLT3*^{WT} 13% *NPM1*^{WT}*FLT3*^{ITD} 70% *NPM1*^{WT}*FLT3*^{WT} 8% *NPM1*^{MUT}*FLT3*^{ITD} 6% *NPM1*^{MUT}*FLT3*^{WT} 11% *NPM1*^{WT}*FLT3*^{ITD} 75% *NPM1*^{WT}*FLT3*^{WT}	*NPM1*^{MUT} but not ITD^{MUT} predicted CR (intensive treated) *NPM1* no effect on OS (intensive and non-intensive) *NPM1*^{MUT}*FLT3*^{WT} relapsed more slowly, improved OS	(Lazenby *et al*, 2014)
156 (+1258)	Intensive	60 (55-83)	33%	17% *NPM1*^{MUT}*FLT3*^{WT} (55-65) 26% *NPM1*^{MUT}*FLT3*^{WT} (>65 years)	*NPM1*^{MUT}*FLT3*^{WT} improved OS in patients 55-65 but not >65 yrs Results confirmed in large validation cohort (1258 patients)	(Ostronoff *et al*, 2015)
301	Intensive	67 (60-85)	43%	28% *NPM1*^{MUT}*FLT3*^{WT}	*NPM1*^{MUT}*FLT3*^{WT} improved OS	(Dickson *et al*, 2016)

Abbreviations: CIR, cumulative incidence relapse; CGN, cytogenetics; CR, complete remission; DFS, disease-free survival; EFS, event-free survival; *FLT3*^{ITD}, *FLT3* with internal tandem duplication; *FLT3*^{WT}, *FLT3* without internal tandem duplication; HSCT, haematopoietic stem cell transplant; IR, intermediate risk cytogenetics; NK, normal karyotype; *NPM1*^{MUT}, *NPM1* mutated; *NPM1*^{WT}, *NPM1* wild-type; OS, overall survival; RFS, relapse-free survival; RR, relapse risk.

chemosensitivity. Some studies have demonstrated a favourable impact of *NPM1*^{MUT} on OS independent of *FLT3*^{ITD} status, whereas others show favourable impact only in the absence of *FLT3*^{ITD}. Studies showing no impact, limited impact, or even a negative impact of *NPM1*^{MUT} contain far fewer patients. In the largest study of intensively treated younger adults, those with *NPM1*^{MUT} in the absence of *FLT3*^{ITD} had the best outcome, those with *NPM1*^{WT}*FLT3*^{ITD} the worst outcome, and patients who were *NPM1*^{MUT}*FLT3*^{ITD} or *NPM1*^{WT}*FLT3*^{WT} had an intermediate prognosis (Gale *et al*, 2008).

The impact of an *NPM1*^{MUT} in children and teenage and young adult patients appears less marked. In older patients, there are conflicting results as to which subpopulations of the elderly derive particular benefit, with one study suggesting no benefit for those >65 years (Ostronoff *et al*, 2015) and another only benefit for those >70 years (Becker *et al*, 2010).

4.1.3.6 Impact of additional cytogenetic lesions in *NPM1*^{MUT}AML

In a study of 631 patients with *NPM1*^{MUT}, cytogenetic lesions were found in almost 15%, with the most frequent chromosomal abnormalities including gain of chromosomes 4, 8, or 21 and loss of chromosome Y or the long arm of chromosome 9 (Haferlach *et al*, 2009). The presence of such chromosomal abnormalities did not appear to alter immunophenotype, gene expression profile, or clinical outcome.

4.1.4 Aims of chapter

The aims of this chapter were:

1. To identify a cohort of patients with AML with pre-treatment biopsy samples available for subsequent IHC analysis of DNA replication licensing proteins and cell cycle status.
2. To define the major prognostic demographic features of this patient cohort, including the cytogenetic, *FLT3*^{ITD}, and *NPM1*^{MUT} status.
3. To define the response to treatment of this cohort.
4. To compare the demographic and outcome data with previously established published

cohorts, with a focus on the UK MRC trials.

4.2 Patients and methods

4.2.1 Identification of patients

Local research ethics approval for the study was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research. Hospital records were reviewed to identify all new cases of AML who presented to UCLH between 1st January 2003 and December 31st 2011. Sources of information included histopathology departmental coding, haematology bone marrow aspirate report logs, pharmacy records, clinic lists, death and discharge summaries, and referrals for allogeneic transplant (HSCT). Pathology records were reviewed to determine patients that had had a diagnostic BMT prior to induction therapy. Patient electronic and paper records were reviewed to define patient demographics including age, sex, presenting WCC, cytogenetics, FAB type, and whether the AML was *de novo*, secondary to a previous haematological disorder (sAML), or therapy-related (tAML).

Details of treatment and outcome were collected. This included information on induction therapy received, induction death, achievement of CR, consolidation or salvage treatment, treatment with HSCT, time to relapse, DFS, and OS. CR was defined as a normocellular BM containing <5% blasts and showing evidence of normal maturation of other BM elements and with a PB neutrophil count of $\geq 1 \times 10^9$/l and a platelet count of $\geq 100 \times 10^9$/l. One patient achieved BM remission with incomplete count recovery but was counted as achieving CR for the purposes of analysis. Remission failures were classified as partial remission (PR, defined as 5%-15% blasts or <5% blasts but with a hypocellular BM), resistant disease (RD, >15% blasts in the BM) or induction death (ID, *i.e.,* related to treatment or hypoplasia). Where evaluation was not possible, deaths within 30 days of entry were classified as ID and deaths later than 30 days after entry as RD. OS was defined as the time from diagnosis to death. For patients achieving CR, DFS was the time from the date of first CR to an event (death in first CR or relapse). Patients who died in remission, for example following HSCT,

were classified as having non-relapse mortality (NRM). Data was censored at death or last follow-up prior to 1st August 2014.

4.2.2. Treatment received

Some patients received treatment as part of one of four national clinical trials that were recruiting during the period of study, AML15 and AML17 for younger adults, and AML14 and AML16 for older adults. The main treatment regimens used in the trials are described in supplementary tables 1 and 2, and examples of clinical trial protocols in supplementary figures 1, 2, and 3.

The AML15 trial recruited 3484 patients, predominantly <60 years of age, between May 2002 and January 2009. The trial was subdivided into the non-APL arm (n=3106) and the APL arm (n=378). In the non-APL arm, a main focus was to compare the induction regimens ADE (Ara-C, daunorubin, etoposide), DA (daunorubicin, Ara-C) and FLAG-Ida (fludarabine, Ara-C, G-CSF, and idarubicin). In addition, patients who had *FLT3*^{ITD} at diagnosis were randomised to recieve, or not, a FLT3 inhibitor (lestaurtinib) after each course of the allocated induction and consolidation chemotherapy. Patients could also be randomised to receive Gemtuzumab ozogamicin (GO). Another key question was the role of HSCT in intermediate-risk patients. For patients with APL, the trial assessed the 'MRC approach' of anthracycline plus Ara-C in combination with ATRA (*All-trans*-retinoic acid) with the 'Spanish approach' that omits Ara-C but has 24 months of ATRA maintenance.

The AML17 trial initially randomised between DA and ADE and between 2 different doses of GO (3mg/m^2 vs 6mg/m^2). However, following the demonstration of superiority of 90mg/m^2 of daunorubicin over lower doses (45mg/m^2) (Fernandez *et al*, 2009), a new randomisation for induction was adopted. This was between DA90 (90mg/m^2 daunorubicin) and DA60 (60mg/m^2 daunorubicin). After course 1, patients were defined by risk of relapse using a validated score (see Table 1.7) based on age, presenting WCC and presence of secondary disease in order to guide whether treatment should be escalated to FLAG-Ida or DClo (daunorubicin and clofarabine), followed by HSCT. In addition, patients with CBF-AML received GO, patients with a *FLT3*

mutation were eligible for lestaurtinib versus placebo randomisation, and non-poor risk patients without CBF-AML or on the lestaurtinib randomisation were eligible for an everolimus versus placebo randomisation. For patients with APL, one of the main focuses of the trial was a randomisation between idarubicin + ATRA versus ATRA + ATO (arsenic trioxide).

The AML14 trial recruited 1593 older patients with AML or high-risk myelodysplasia (MDS) between December 1998 and March 2006 and had both intensive and non-intensive treatment approaches. In the intensive arm, there were two main randomisation questions in the context of induction treatment with DAT chemotherapy (daunorubicin, Ara-C, thioguanine): firstly, to compare a daunorubicin dose of 35mg/m^2 (DAT35) with 50mg/m^2 (DAT50), and secondly to evaluate an increased daily dose of Ara-C (200mg/m^2 compared to 400mg/m^2). In addition, patients receiving the DAT35 option could be randomised to receive the cyclosporin analogue and p-glycoprotein inhibitor PSC-833, a drug that delays the excretion of daunorubicin. The non-intensive arm of the trial initially compared LDAC (low dose Ara-C) with or without ATRA with best supportive therapy. This approach ended prematurely as survival was significantly better in the LDAC-treated cohort. Between June 2004 and March 2006, 108 patients were recruited to the non-intensive arm and randomised between LDAC and LDAC, with the addition of 5mg of GO on day one of each course.

The AML16 trial also had both intensive and non-intensive arms. In the intensive part of the study, patients were randomised between induction with DA50 or DClo, with a further GO randomisation available in both arms. For patients who were in remission after 2 cycles of induction treatment, there was an option for randomisation between no treatment or a further cycle of DA. Following this, patients could undergo a reduced-intensity HSCT or could be further randomised between no further therapy or demethylation treatment with azacytidine. The non-intensive arm of the trial followed a 'pick-a-winner' format (Hills & Burnett, 2011) where LDAC treatment had a GO randomisation but also a number of other agents including ATO, clofarabine, and the farnesyltransferase inhibitor tipifarnib.

For patients who were not enrolled onto one of the national clinical trials, the general intensive approach was remission induction using DA50 followed by a further cycle of

DA50 for patients achieving response, and for patients not proceeding to allogeneic transplant, consolidation cycles of MACE (amsacrine, Ara-C, etoposide) and MidAC (mitoxantrone, Ara-C), or high dose Ara-C (1.5g/m^2). For patients receiving non-intensive treatment, the general approach was LDAC with GO where supply of the drug permitted.

Where donor availability permitted, patients with relapsed disease, high-risk disease according to AML17 score, high risk-cytogenetics, or intermediate-risk cytogenetics in the absence of an isolated *NPM1* mutation proceeded to HSCT. In general, patients aged ≤40 years received myeloablative conditioning with cyclophosphamide and total body irradiation (Cy/TBI) for sibling donors, and with the addition of fludarabine (Flu/Cy/TBI) where the donor was matched-unrelated (MUD) or mismatched-unrelated (MMUD). A T-deplete strategy was used with alemtuzumab in the stem cell product prior to infusion. For most patients >40 years or with comorbidity, a reduced-intensity approach using fludarabine, melphalan and *in vivo* alemtuzumab was used.

4.2.3 Molecular analysis

Molecular analysis was performed on DNA extracted from diagnostic samples extracted as described in chapter 2.

4.2.3.1 Screening and quantification of *FLT3*^{ITD}

Patient mutational status for *FLT3* (*FLT3*^{ITD} vs *FLT3*^{WT}) was determined by PCR amplification of the juxtamembrane domain of *FLT3* using sequence-specific forward and reverse primers as described in chapter 2. The primer sequences and annealing temperature are shown in Table 4.4. The larger PCR product of a *FLT3*^{ITD} was seen as a slower moving band (Figure 4.1), and all patient samples with such a band or bands were scored as *FLT3*^{ITD} positive.

Table 4.4 Primers and annealing temperatures used to assess *NPM1*^{MUT} and *FLT3*^{ITD} status and percentage mutant alleles.

Exon/Domain	Primer	Sequence	Annealing temperature (^{o}C)
FLT3 exons 14 and 15 (including intervening intron)	FLT3ex11/F FLT3ex12/R	5'-GCAATTTAGGTATGAAAGCCAGC-3' 5'-CTTTCAGCATTTTGACGGCAACC- 3'	62
FLT3 exons 14 and 15 (including intervening intron)	FLT3ex11/F FLT3ex12/R*	5'-GCAATTTAGGTATGAAAGCCAGC-3' 5'-CTTTCAGCATTTTGACGGCAACC- 3'	62
NPM1 exon 12	NPMex12/F NPMex12/R*	5' -CTTAACCACATTTCTTTTTTTTTTTTCCAG- 3' 5' -GGACAACATTTATCAAACACGGTAG- 3'	62

* indicates fluorescently-labelled primer

For quantification of *FLT3*^{ITD} mutant level or where DNA sample quality was poor, PCR amplification was carried out as before except using a fluorescently-labelled forward primer, 25 cycles of PCR, and analysed using the CEQ^{TM} 8000 Genetic Analysis System as described in chapter 2. The primer sequences and annealing temperature are shown in Table 4.4. A representative analysis is shown in Figure 4.2.

4.2.3.2 Screening and quantification of *NPM1* mutations

The presence of an *NPM1* mutation was determined by amplification of *NPM1* exon 12 using sequence-specific primers with a fluorescently-labelled reverse primer (Table 4.4), and 28 cycles of PCR, and analysed using the CEQ^{TM} 8000 Genetic Analysis System as described in chapter 2. A representative analysis is shown in Figure 4.2.

4.2.4 Statistical analysis

The comparison of continuous variables between 2 groups was performed using the Mann-Whitney U test. The comparison of continuous variables across multiple groups was performed using the Kruskal-Wallis test. Contingency data between 2 groups was assessed using either the Chi-square or Fisher's exact test as appropriate. Where a trend was being assessed over multiple groups, the Chi-square test for trend was employed. Correlation was assessed using Spearman's rank correlation coefficient. OS and DFS were assessed using the method of Kaplan-Meier, and the difference between groups by the log-rank (Mantel-Cox) test. All tests were two-tailed. All the above statistics were performed using Graphpad Prism version 6 (GraphPad Software, Inc., California, USA). Multivariate analysis was performed using multinomial logistic regression and SPSS version 22 (IBM, New York, USA). The values for CIR and NRM were calculated using the competing risks method (Scrucca *et al*, 2007) using 'R' version 3.2.1 (www.r-project.org/). A P value <0.5 was considered statistically significant.

4.3 Results

4.3.1 Characteristics of patient cohort

In total, 202 patients with a new diagnosis of AML made between January 2003 and December 2011, and in whom trephine biopsies were taken prior to treatment, were identified. In 15 cases the sample block could not be located in the tissue archive. A further six patients were subsequently excluded either because there was inadequate material for further IHC (n=5), or because of a coincident infiltration with a B-cell malignancy, leaving a total cohort of 181 patients. The number of patients accrued per year of the study is shown in Figure 4.3. Fifteen patients (8%) had APL and 166 had other forms of AML. There was a trend towards patients with APL being younger; median age 44 years vs 56 years in the non-APL patients (P=0.07). The frequency of APL and other cytogenetic subtypes in the 94 patients aged <60 years with available cytogenetic data was broadly similar to the frequencies seen in the large MRC data set of younger adults (Table 4.5), the main difference being an increase in patients with adverse cytogenetics (20% vs 10%) and a decrease in those with an intermediate-risk abnormal karyotype (7% vs 25%).

The main demographic features for the non-APL patients are shown in Table 4.6. Just over half of the patients (57%) were aged <60 years. There was no difference in sex distribution between the older (≥60 years) and younger cohorts. Patients in the younger cohort were more likely to have *de novo* disease and a significantly higher presenting WCC (median 22.5 vs 9 x 10^9/l respectively, P=0.04). Even excluding the APL patients, there were fewer patients with favourable-risk cytogenetics in the older cohort (4% vs 16%, P=0.03), and a trend towards a greater number of patients with failed cytogenetics (22% vs 11%, P=0.05).

The main demographic features for the APL patients are shown in Table 4.7. As indicated above, there was a trend towards younger patients in the APL cohort, with 12 (80%) aged <60 years at diagnosis. One patient had secondary disease following chemotherapy for a prior malignancy.

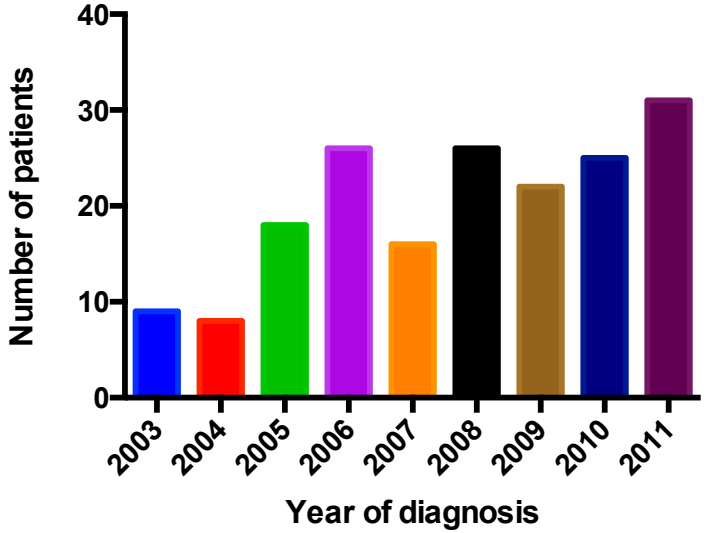


Figure 4.3 The number of patients identified with suitable bone marrow biopsy samples available for further analysis, grouped by year of diagnosis.

Table 4.5 Comparison between frequency of cytogenetic lesions in the MRC cohort and the UCLH cohort with available cytogenetic data.

Risk group	Refined MRC classification*	Proportion of patients in MRC cohort (n=5876)	Proportion of all patients in UCLH cohort (n=151)	Proportion of patients <60 years in UCLH cohort (n=94)	Proportion of patients ≥60 years in UCLH cohort (n=57)
Favourable	t(15;17)	12%	10%	13%	5%
	t(8;21)	8%	5%	7%	2%
	inv(16) or t(16;16)	3%	5%	6%	2%
Intermediate	Normal Karyotype	42%	48%	45%	51%
	Other non-complex	25%	13%	7%	23%
Adverse		10%	19%	20%	18%

*Classified according to (Grimwade *et al*, 2010)

Table 4.6 Presenting features of patients with non-APL AML

	All patients	<60 years	≥60 years	P
No. patients (%)	166 (100)	94 (57)	72 (43)	
Median age, years (range)	56 (13-86)	41.5 (13-59)	69 (60-86)	
Sex, (%)				0.27
Male	90 (54)	47 (50)	43 (60)	
Female	76 (46)	47 (50)	29 (40)	
Type of AML (%)				0.02 (*de novo* vs sAML/tAML)
De Novo	136 (82)	83 (88)	53 (74)	
Secondary	19 (11)	4 (4)	15 (21)	
Therapy-related	11 (7)	7 (7)	4 (6)	
Median WCC x 10^9/l (range)	17 (0-385)	22.5 (0-385)	9 (1-301)	0.04
FAB type, no. (%)				
M0	12 (7)	6 (6)	6 (8)	
M1	39 (23)	26 (28)	13 (18)	
M2	65 (39)	35 (37)	30 (42)	
M4	17 (10)	11 (14)	6 (8)	
M5	25 (15)	12 (13)	13 (18)	
M6	2 (1)	1 (1)	1 (1)	
M7	1 (<1)	1 (1)	0 (0)	
Unknown	5 (3)	2 (2)	3 (4)	NS for all
Cytogenetics (% all/known)				
Favourable	15 (9 /11)	13 (14/16)	2 (3 /4)	0.03
Intermediate	92 (55/68)	50 (53/61)	42 (58/78)	0.06
NK	76 (46/56)	43 (46/52)	29 (39/54)	0.99
Adverse	29 (17/21)	19 (20/23)	10 (14/19)	0.67
Failed	30 (16/-)	10 (11/-)	16 (22/-)	0.05
Unknown	4 (2/-)	2 (2/-)	2 (3/-)	0.99

Abbreviations: NK, normal karyotype; sAML, secondary AML; tAML; treatment-related AML

Table 4.7 Presenting features of patients with APL

	All patients	**Patients <60 years**	**Patients ≥60 years**
Number (%)	15 (100)	12 (80)	3 (20)
Median age, years (range)	44 (16-70)	42 (15-66)	64 (64-70)
Median WCC x 10^9/l (range)	3 (0-99)	4.5 (0-99)	2 (1-4)
Sex (%) Male Female	 8 (53) 7 (47)	 6 (50) 6 (50)	 2 (67) 1 (33)
Type of AML (%) *De Novo* Secondary/Treatment	 14 (93) 1 (7)	 11 (92) 1 (8)	 3 (100) 0 (3)

4.3.2 Presenting features according to cytogenetic risk group in non-APL patients

The presenting clinical features according to cytogenetic risk group are shown in Table 4.8. Patients with favourable-risk cytogenetics had the youngest median age (44 years), followed by patients in the adverse, intermediate, and failed/unknown cytogenetic groups (52, 57, and 62.5 years respectively, P=0.03, Kruskal-Wallis test across all groups). This remained significant when patients with failed/unknown cytogenetics were excluded (P=0.03). The intermediate-risk group had the highest presenting WCC (median 28 x 10^9/l), followed by the favourable, adverse, and failed/unknown groups (medians 21, 11, and 2.5 x 10^9/l respectively, P=0.008). However, when the failed/unknown group was excluded there was no significant difference across the favourable, intermediate, and adverse groups (P=0.13). The adverse-risk group had a greater proportion of male patients (72%), but this did not reach statistical significance when compared to the proportions seen in the other cytogenetic risk groups (P=0.06). There were no patients with secondary AML in the favourable group compared with 10%, 14%, and 20% in the intermediate, adverse, and failed/unknown groups respectively, but this did not reach statistical significance.

4.3.3 Presenting features according to molecular risk group in the non-APL cohort

FLT3^{ITD} status was available in 151 non-APL patients. Thirty-one (21%) had a *FLT3*^{ITD}, with the majority of these (n=18, 58%) having a intermediate mutant level (25-50%) (Table 4.9). However, there was a broad range of mutant levels suggesting that there were patients with minor clones with heterozygous mutations (i.e. <25% mutant) (although sample impurity cannot be excluded), as well as patients with >50% mutational burden indicative of homozygous mutations in at least some of the cells (Figure 4.4). The median *FLT3*^{ITD} burden was 37% (range 5-93%). The frequency of *FLT3*^{ITD} patients in the *FLT3*$^{ITD\text{-}LOW}$, *FLT3*$^{ITD\text{-}INT}$, and *FLT3*$^{ITD\text{-}HIGH}$ groups were 23%, 58%, and 19% respectively (Table 4.9). The equivalent figures for an, albeit younger, population of patients from the MRC trials were 29%, 56% and 15% (Gale *et al*, 2008). Thus, the general distribution of *FLT3*^{ITD} burden seen in the UCLH cohort, with the greatest number of patients having a mutant burden of 25-50%, was consistent with other studies.

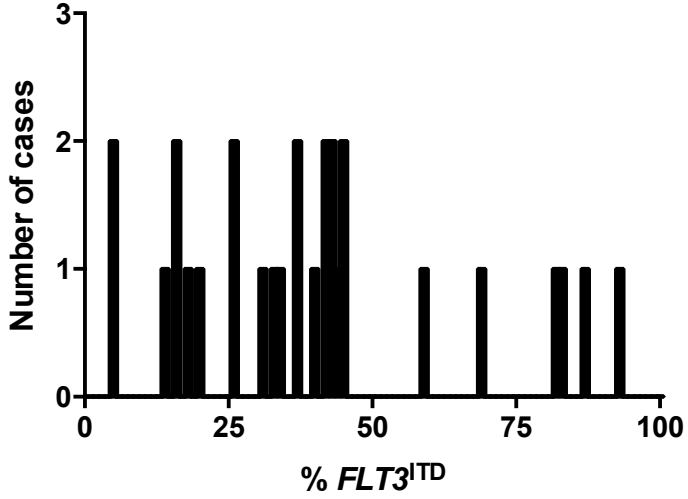


Figure 4.4 The total *FLT3*^{ITD} mutant allele level in 31 *FLT3*^{ITD} patients.

Table 4.8 Presenting clinical features according to cytogenetic risk group in non-APL patients

	Favourable (non-APL)	Intermediate	Adverse	Failed/unknown	P
Number (% total/known)	15 (9/11)	92 (55/68)	29 (17/21)	30 (18)	
Median age, years (range)	44 (16-64)	57 (13-86)	52 (13-81)	62.5 (18-83)	0.03 (0.03 excluding failed/unknown cases)
Sex (%)					
Female	6 (40)	44 (48)	8 (28)	15 (50)	
Male	9 (60)	48 (52)	21 (72)	15 (50)	NS for all
Median WCC x 10^9/l (range)	21 (3-47)	28 (1-385)	11 (0-346)	2.5 (1-205)	0.008 (0.13 excluding failed /unknown cases)
AML type (%)					
De novo	13 (87)	80 (87)	21 (72)	22 (73)	
Secondary	0 (0)	9 (10)	4 (14)	6 (20)	
Treatment	2 (13)	3 (3)	4 (14)	2 (7)	NS for all

Table 4.9 Presenting features in the patients with non-APL disease and known *FLT3*^{ITD} status

	Total (%)	*FLT3*^{WT} (%)	*FLT3*^{ITD} (%)	P	*FLT3*$^{ITD-LOW}$ (%)	*FLT3*$^{ITD-INT}$ (%)	*FLT3*$^{ITD-HIGH}$ (%)	P
Total (%)	151 (100)	120 (79)	31 (21)		7 (5)	18 (12)	6 (4)	
Type of AML (%)								0.29
De Novo	125 (83)	96 (80)	29 (94)		6 (86)	17 (94)	6 (100)	
Secondary	17 (11)	15 (13)	2 (6)		1 (14)	1 (6)	0 (0)	
Treatment	9 (6)	9 (8)	0 (0)	0.11	0 (0)	0 (0)	0 (0)	
FAB type (%)								
M0	11 (7)	10 (8)	1 (3)	0.46	0 (0)	1 (6)	0 (0)	0.96
M1	38 (25)	22 (18)	16 (52)	0.0005	2 (29)	11 (61)	3 (50)	0.4
M2	62 (41)	56 (47)	6 (19)	0.007	3 (43)	3 (17)	0 (0)	0.05
M4	15 (10)	10 (8)	5 (16)	0.31	2 (29)	1 (6)	2 (33)	0.9
M5	20 (13)	17 (14)	4 (13)	0.99	0 (0)	2 (11)	1 (17)	0.3
M6	2 (1)	2 (2)	0 (0)	0.99	0 (0)	0 (0)	0 (0)	0.99
M7	0 (0)	1 (1)	0 (0)	0.99	0 (0)	0 (0)	0 (0)	0.99
Unknown	2 (1)	2 (2)	0 (0)	0.99	0 (0)	0 (0)	0 (0)	0.99
Sex (%)								
Female	70 (46)	54 (45)	16 (52)		5 (71)	9 (50)	2 (33)	0.17
Male	81 (54)	66 (55)	15 (48)	0.55	2 (29)	9 (50)	4 (67)	
Median age, years (range)	56 (13-86)	57 (13-86)	54 (13-81)	0.46	58 (20-81)	54 (13-73)	53 (14-61)	0.6
Median WCCx10^9/l (range)	17 (0-385)	12 (0-346)	58 (2-385)	<0.0001	23 (2-180)	62.5 (8-231)	103.5 (35-385)	0.12
Cytogenetics (% all/known)								
Favourable	14 (9/11)	12 (10/12)	2 (6/7)	0.74	1 (14/17)	1 (6/6)	0 (0/0)	
Intermediate	85 (56/66)	60 (50/61)	25 (81/86)	0.01	5 (71/83)	15 (83/83)	5 (83/83)	
Normal	69 (46/54)	46 (38/46)	23 (74/79)	0.003	3 (43/50)	15 (83/83)	5 (83/83)	
Adverse	25 (17/20)	23 (19/23)	2 (6/7)	0.06	0 (0/0)	1 (6/6)	1 (17/17)	
Failed	24 (16/-)	22 (18/-)	2 (6/-)	0.17	1 (14/-)	1 (6/-)	0 (0/-)	NS
Unknown	3 (2/-)	3 (3/-)	0 (0/-)	0.99	0 (0/-)	0 (0/-)	0 (0/-)	for all

Abbreviations: *FLT3*$^{ITD-LOW}$, <25% mutant alleles; *FLT3*$^{ITD-INT}$, 25-50% mutant alleles; *FLT3*$^{ITD-HIGH}$, >50% mutant alleles; NS, non-significant.

The frequency of *FLT3*^{ITD} in the intermediate karyotype group was 30%. A higher percentage of *FLT3*^{ITD} compared to *FLT3*^{WT} patients had an intermediate karyotype (86% vs 61%, P=0.01) and normal karytoype (NK) (79% vs 46%, P=0.003), and a non-statistically significant decrease in sAML/tAML. They also had a higher presenting WCC, with a relative increase in WCC with mutational burden (r=0.4, 95% CI 0.05-0.67, P=0.025) (Figure 4.5). *FLT3*^{ITD} patients were also more likely than *FLT3*^{WT} patients to be FAB type M1 (52% vs 18%, P=0.0005) and less likely to be M2 subtype (19% vs 47%, P=0.007). There was no difference in age or sex distribution. Many of these presenting features have been demonstrated in large published cohorts. These include the negative association with M2 FAB type (Thiede *et al*, 2002) and the positive association with intermediate-risk and NK cytogenetics, and increased WCC (Kottaridis *et al*, 2001; Gale *et al*, 2008).

A total of 49 of the 150 patients non-APL patients in whom *NPM1* status was available (33%) had *NPM1*^{MUT}, and this proportion rose to 49% and 58% respectively when only the intermediate karyotype or NK patients were considered (Table 4.10). In fact, only 2 patients with known karyotype and *NPM1*^{MUT} were not in the intermediate risk group. The percentage of patients with *NPM1*^{MUT} in the cohort overall, and in the intermediate and NK cytogenetic groups, were broadly in keeping with the large published cohorts. For example, in the MRC cohort (Gale *et al*, 2008) the proportion of patients with *NPM1*^{MUT} overall, in the intermediate and NK groups was 41%, 51%, and 62% respectively.

Patients with *NPM1*^{MUT} were less likely to have M2 FAB type compared to *NPM1*^{WT} (29% vs 48%, P=0.02), with a non-significant increase in M1 (33% vs 21%, P=0.16) and M4 (14% vs 8%, P=0.26), and a significant increase in M5 (22% vs 9%, P=0.04). *NPM1*^{MUT} patients were more likely to have *de novo* than secondary or treatment-related AML than *NPM1*^{WT} patients (P=0.04). Although there were more females than males with an *NPM1* mutation, overall (55% vs 45%), this did not reach statistical significance. There was no difference in age between *NPM1*^{WT} and *NPM1*^{MUT} patients, but the latter had a significantly higher presenting WCC, median 54 vs 8 x 10^9/l (P<0.0001). In contrast to *FLT3*^{ITD}, the mutant burden was far less variable (Figure 4.6A), with a median of 46% (range, 19-54%), in keeping with the majority of cells being heterozygous for the mutation. In fact, only 4% of patients

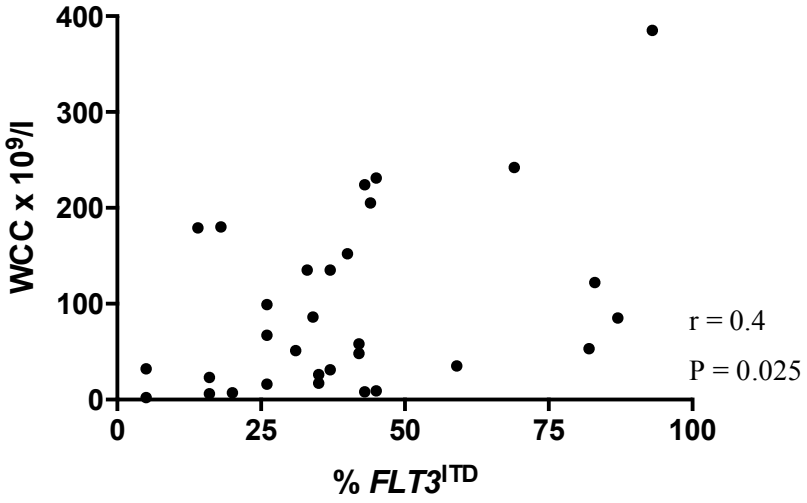


Figure 4.5 Correlation between *FLT3*^{ITD} burden and presenting WCC in 31 *FLT3*^{ITD} patients.

Table 4.10 Presenting features in the patients with non-APL disease and known *NPM1* status

	Total (%)	*NPM1*^{WT} (%)	*NPM1*^{MUT} (%)	P
Total (%)	150 (100)	101 (67)	49 (33)	
Type of AML (%)				0.04
De Novo	124 (83)	79 (78)	45 (92)	
Secondary	17 (11)	15 (15)	2 (4)	
Treatment	9 (6)	7 (7)	2 (4)	
FAB type (%)				
M0	11 (7)	10 (10)	1 (2)	0.1
M1	37 (25)	21 (21)	16 (33)	0.16
M2	62 (41)	48 (48)	14 (29)	0.02
M4	15 (10)	8 (8)	7 (14)	0.26
M5	20 (13)	9 (9)	11 (22)	0.04
M6	2 (1)	2	0	0.99
M7	1 (1)	1	0	0.99
Unknown	2 (1)	2	0	0.99
Sex (%)				0.17
Female	70 (47)	43 (43)	27 (55)	
Male	80 (53)	58 (57)	22 (45)	
Median age, years (range)	56 (13-86)	59 (13-86)	54 (13-77)	0.76
Median WCC x 10^9/l (range)	18 (0-385)	8 (0-346)	54 (2-385)	<0.0001
Cytogenetics (% all/known)				
Favourable	14 (9/11)	14 (14/18)	0 (0/0)	0.002
Intermediate	85 (57/69)	43 (43/54)	42 (86/95)	<0.0001
Normal	69 (46/56)	29 (29/36)	40 (82/91)	<0.0001
Adverse	25 (17/20)	23 (23/29)	2 (4/5)	0.0009
Failed	23 (15/-)	18 (18/-)	5 (10/-)	0.24
Unknown	3 (2/-)	3 (3/-)	0 (0/-)	0.55
***FLT3* status (%)**				
FLT3^{WT}	119 (79)	91 (90)	28 (57)	
FLT3^{ITD}	31 (21)	10 (10)	21 (43)	<0.0001
***FLT3*^{ITD} level (% of *FLT3*^{ITD})**				
ITD-LOW	7 (23)	5 (50)	2 (10)	
ITD-INT	18 (58)	4 (40)	14 (67)	
ITD-HIGH	6 (19)	1 (10)	5 (24)	0.03

Abbreviations: ITD-LOW, <25% mutant alleles; ITD-INT, 25-50% mutant alleles; ITD-HIGH, >50% mutant alleles.

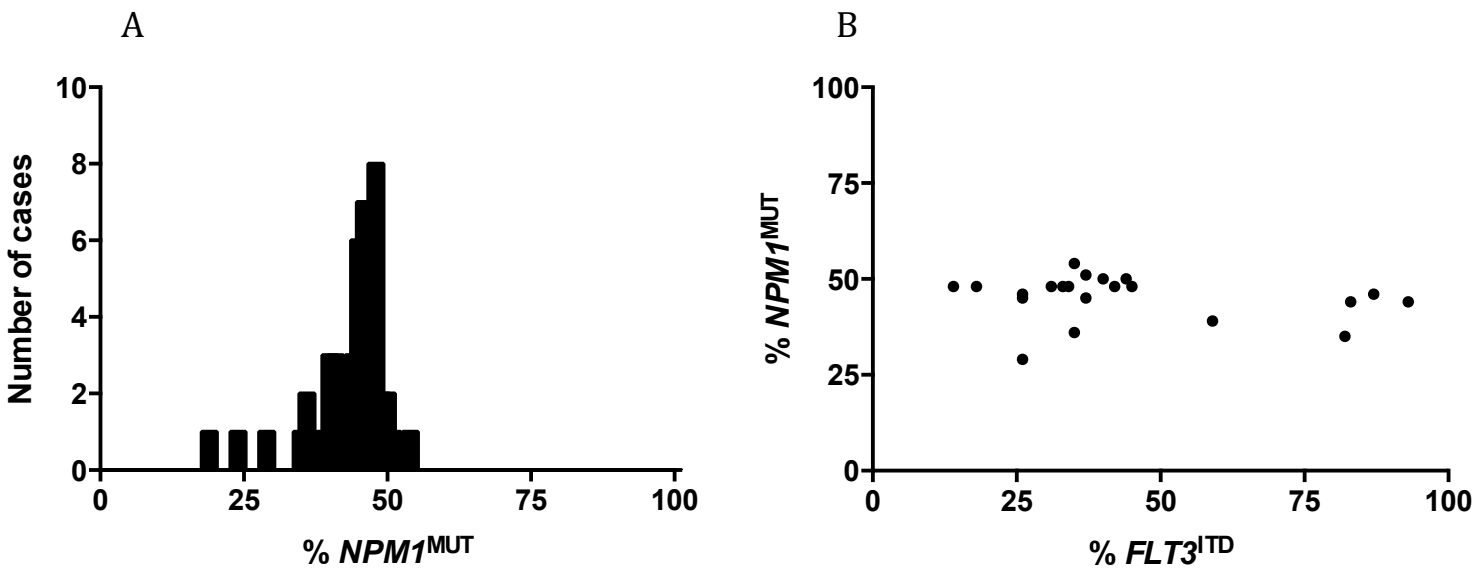


Figure 4.6 The mutant level and relationship to *FLT3*^{ITD} in *NPM1*^{MUT} patients. (A) The distribution of *NPM1* mutant burden in 49 *NPM1*^{MUT} patients. (B) The relative mutant burden of *FLT3*^{ITD} and *NPM1*^{MUT} in the 21 patients with both mutations.

had *NPM1* mutant level <25% and 4% >50%. This lack of variability was particularly striking when relative mutant burden was compared for the 21 patients harbouring both an *NPM1*^{MUT} and a *FLT3*^{ITD} (Figure 4.6B). This decreased variability mirrors that seen in the MRC data for *NPM1*^{MUT} with a median of 43% (range 6-78%), where only 8% of *NPM1*^{MUT} had levels <25%, and 3% >50% (Gale *et al*, 2008).

The frequency of the combined *NPM1/FLT3* genotypes for the non-APL cohort for *NPM1*^{MUT}*FLT3*^{ITD}, *NPM1*^{MUT}*FLT3*^{WT}, *NPM1*^{WT}*FLT3*^{ITD}, and *NPM1*^{WT}*FLT3*^{WT} genotypes was 14%, 19%, 7%, and 61% respectively. The equivalent figures for the MRC cohort were 17%, 24%, 11%, and 47%. This higher frequency of *NPM1*^{WT}*FLT3*^{WT} genotype may reflect the larger number of patients with adverse cytogenetics in the UCLH cohort, as when only the intermediate-risk patients were considered the frequency of the respective genotypes was 16%, 21%, 7%, and 29%.

Of interest is the increasing likelihood of having an *NPM1* mutation where there is a higher *FLT3*^{ITD} allele burden (Table 4.10). This was previously recognised in the MRC studies (Gale *et al*, 2008) where the frequency of patients with *NPM1*^{MUT} in the *FLT3*^{WT}, *FLT3*$^{ITD\text{-}LOW}$, *FLT3*$^{ITD\text{-}INT}$, and *FLT3*$^{ITD\text{-}HIGH}$ groups was 34%, 51%, 59%, and 81% respectively (P<0.0001). The equivalent figures in the UCLH cohort were 24%, 29%, 78%, and 83%, with an association between *NPM1*^{MUT} and *FLT3*^{ITD} (P<0.001), and a trend for increased percentage of patients with *NPM1*^{MUT} with increasing *FLT3*^{ITD} level (P=0.03).

4.3.4 Treatment

Overall, 140 non-APL patients (84%) were known to have received intensive treatment, with higher rates in patients aged <60 years (96%) than in those ≥60 years (69%) (P<0.0001). Patients aged <60 years were also more likely to have received their induction treatment in the context of a national clinical trial (P=0.015) (Table 4.11). An overview of the frequencies of the different regimens used is shown in Table 4.12, with the majority of patients receiving DA50. A further three patients had intensive-style treatment with high-dose or intermediate dose Ara-C but

Table 4.11 Overall treatment approach in the patients with non-APL disease

	All patients	Patients <60 years	Patients ≥60 years
Number of patients (%)	166 (100)	94 (57)	72 (43)
Clinical Trial, no. (%)	94 (57)	62 (66)	32 (44)
14	8 (5)	0 (0)	8 (11)
15	47(28)	42 (45)	5 (7)
16	18 (11)	0 (0)	18 (25)
17	21 (13)	20 (21)	1 (1)
Treated Off trial	72 (43)	32 (34)	40 (56)
Treatment (%)			
Int	140 (84)	90	50
Int (no anthracycline)	3 (2)	2	1
Non-Int	17 (10)	1	16
No treatment/UK	6 (4)	1	5

Abbreviations: Int, intensive treatment; Non-Int, non-intensive treatment; UK, unknown

Table 4.12 Initial induction regimens used in the 140 intensively treated non-APL patients

Intensive Regimen	Number of patients (%)	Also received ATRA (%)	Also received GO (%)
DA	99 (71)	2 (1)	12 (9)
DA50	97 (69)		
DA60	1 (<1)		
DA90	1 (<1)		
ADE	31 (22)	1 (<1)	4 (3)
FLAG-Ida	7 (5)	0 (0)	3 (2)
DClo	3 (2)	0 (0)	1 (<1)

Abbreviations: DA, cytarabine and 50, 60, or 90 mg/m^2 daurobicin; ADE, cytarabine, duanorubicin, etopsoide; FLAG-Ida, fludarabine, cytarabine, idarubicin, G-CSF; DClo, daunorubicin, clofrabine; GO, gemtuzumab ozogamicin. Details of regimens are given in supplementary table 1.

without an anthracycline as part of their initial induction treatment because of cardiac concerns in the acute setting. These patients were not included in the analysis of the intensively treated patients. Seventeen patients received non-intensive therapy. A further five patients elected to receive supportive care, with one patient electing to return to their native country prior to commencing therapy (treatment unknown) and being lost to follow-up. Thus, in keeping with the MRC trials as a whole, the majority of patients (91%) were treated with either DA50 or ADE, indicating that the treatment regimens used in the UCLH cohort effectively matched the predominant regimens used in the AML15 trial and the initial version of the AML17 trial. There is little evidence of a clinical outcome difference for using ADE over DA50.

The majority of APL patients, overall and in both age cohorts, had their initial treatment in the context of a national clinical trial (overall, 9 out of 15 patients, 60%). All patients received ATRA in combination with an anthracycline with or without Ara-C apart from one patient who received ATRA-ATO as part of the AML17 trial.

4.3.5 Response to intensive induction treatment

Of the 140 intensively treated non-APL patients, 17 (12%) died in the first 30 days and were labelled as ID. Compared to those who survived beyond 30 days (regardless of remission status), they had a higher median presenting WCC (median 62 vs 16 x 10^9/l, P=0.02) and were significantly older (median 64 vs 52 years, P=0.002) (Table 4.13). There were no demonstrable differences with respect to sex, *de novo* disease or cytogenetic risk group. These results were in keeping with a multicentre study showing that the most important predictors for early death (first four weeks in this study) are age and performance status, but that a high presenting WCC (>100 x 10^9/l) is also associated with ID (Walter *et al*, 2011).

For patients who survived beyond 30 days, the features of those achieving CR following one cycle of intensive induction therapy and at any time are presented in

Table 4.13 Features of intensively treated non-APL patients who did or did not die in the first 30 days

	ID (death in first 30 days)	**Not ID (survived beyond 30 days)**	**P**
Patient no. (%)	17 (12)	123 (88)	
Median age, years (range)	64 (18-81)	52 (13-76)	0.002
Sex (%) Male (%) Female (%)	 10 (59) 7 (41)	 64 (52) 59 (48)	0.8
Type of AML (%) De Novo (%) Secondary/Treatment (%)	 12 (71) 5 (29)	 107 (87) 16 (	0.14
Median WCC x 10^9/l (range)	62 (2-376)	16 (0-385)	0.02
Cytogenetics (% all/known) Favourable Intermediate NK Adverse Failed/Unknown	 1 (6) 10 (74) 6 (35) 1 (6) 5 (29)	 12 (10) 70 (57) 58 (47) 22 (18) 19 (15)	 0.99 0.34 0.77 0.46 0.17

Abbreviation: ID, induction death

Table 4.14 Response to first induction treatment for intensively treated patients surviving beyond 30 days

	RD post cycle 1	CR post cycle 1	P
Patient no. (%)	31(22)	92 (66)	
Median age, years (range)	57 (14-74)	50.5 (13-76)	0.16
Sex (%) Male Female	 12 (39) 19 (61)	 46 (50) 46 (50)	 0.3
Type of AML (%) *De Novo* Secondary/Treatment	 28 (90) 3 (10)	 79 (86) 13 (14)	 0.76
Median WCC x 10^9/l (range)	8 (1-385)	21.5 (0-231)	0.23
Cytogenetics No. (% all/% known) Favourable Intermediate NK Adverse Failed/Unknown	 0 (0/0) 12 (39/48) 12 (39/48) 13 (42/52) 6 (19/-)	 12 (13/15) 58 (63/80) 46 (50/63) 9 (7/9) 13 (11/-)	 0.07 0.01 0.49 <0.0001 0.6

Abbreviation: RD, resistant disease

Table 4.15 Presenting features of intensively treated patients who did or did not attain remission at any time

	CR never achieved	CR achieved	P
Patient no. (%)	22 (16)	101 (72)	
Median age, years (range)	59 (14-73)	50 (13-76)	0.06
Sex (%) Male Female	 8 (36) 14 (64)	 51 (50) 50 (50)	0.25
Type of AML (%) *De Novo* Secondary/Treatment	 19 (86) 3 (14)	 88 (87) 13 (13)	0.99
Median WCC x 10^9/l (range)	7.5 (1-385)	21 (0-346)	0.22
Cytogenetics (% all/known) Favourable Intermediate NK Adverse Failed/Unknown	 0 (0/0) 7 (32/41) 7(32/41) 10 (45/59) 5 (23/-)	 12 (12/14) 63 (62/72) 51 (50/59) 12 (12/14) 14 (14/-)	 0.36 0.02 0.29 0.0002 0.33

Tables 4.14 and 4.15 respectively. Patients with PR and refractory disease were grouped within remission failures. There were no significant differences between the groups with respect to sex, presenting WCC, or *de novo* disease. Patients failing CR following one cycle were non-significantly older (Table 4.14) and this age difference approached significance when patients achieving CR at any time were considered (Table 4.15). The group that never attained remission had a significantly greater proportion of patients with adverse-risk cytogenetics (59% vs 14%, P=0.0002) (Table 4.15).

Response to induction therapy was assessed according to *NPM1* (n=128) and *FLT3* (n=129) mutational status (Table 4.16). Compared to $NPM1^{WT}$, $NPM1^{MUT}$ patients were significantly associated with achievement of CR after course 1 (84% vs 61%, P=0.009), although this significance was lost for those achieving CR at any point because of the increased proportion of $NPM1^{WT}$ patients that went into CR after further courses (89% v 71%, P=0.08). For those patients with intermediate karyotype and known molecular status (n= 73 for both *NPM1* and *FLT3*), $NPM1^{MUT}$ status was associated with a significantly greater chance of achieving remission, both after one cycle of treatment (86% vs 56%, P=0.005) and at any time (89% vs 67%, P=0.03) (Table 4.17). $FLT3^{ITD}$ did not influence the achievement of CR in either the whole intensively treated cohort or in patients with intermediate-risk cytogenetics. However, when the combined *NPM1/FLT3 g*enotype was assessed, the lowest CR rate after one cycle of intensive treatment was seen in the $NPM1^{WT}FLT3^{ITD}$ patients, both in the non-APL cohort as a whole (50%, P=0.02), and the intermediate-risk cytogenetic group (29%, P=0.009). There were no significant differences seen in the numbers who eventually attained remission after subsequent rounds of treatment. This association between *NPM1* status but not *FLT3* status with the achievement of remission following intensive induction treatment mirrors that seen in the majority of the large published cohorts described in Tables 4.1 and 4.3, including the studies of patients treated on MRC trials (Kottaridis *et al*, 2001; Gale *et al*, 2008).

To assess whether characteristics in the UCLH cohort found to be associated with failure to achieve CR, either because of ID or RD, were independent factors for predicting response to therapy, these factors were entered into a multivariate

Table 4.16 Response to intensive induction treatment in non-APL patients according to genotype.

	***NPM1*^{WT} (n=85)**	***NPM1*^{MUT} (n=43)**	**P***	***FLT3*^{WT} (n=100)**	***FLT3*^{ITD} (n=29)**	**P***	***NPM1*^{WT} *FLT3*^{WT} (n=75)**	***NPM1*^{MUT} *FLT3*^{WT} (n=24)**	***NPM1*^{WT} *FLT3*^{ITD} (n=10)**	***NPM1*^{MUT} *FLT3*^{ITD} (n=19)**	**P***
Status after course 1											
CR (%)	52 (61)	36 (84)		64 (64)	21 (72)		43 (57)	20 (83)	5 (50)	16 (84)	0.02
RD (%)	24 (28)	3 (7)		25 (25)	6 (21)		22 (29)	1 (4)	4 (40)	2 (11)	
ID (%)	9 (11)	4 (9)	0.009	11 (11)	2 (7)	0.5	10 (13)	3 (13)	1 (10)	1 (5)	
CR ever	60 (71)	33(89)	0.08	70 (70)	24 (83)	0.2	48 (64)	21 (88)	8 (80)	16 (84)	0.07

*P values refer to attaining CR vs not attaining CR.

Table 4.17 Response to intensive induction treatment according to genotype in patients with intermediate-risk cytogenetics.

	***NPM1*^{WT} (n=36)**	***NPM1*^{MUT} (n=37)**	**P***	***FLT3*^{WT} (n=50)**	***FLT3*^{ITD} (n=23)**	**P***	***NPM1*^{WT} *FLT3*^{WT} (n=29)**	***NPM1*^{MUT} *FLT3*^{WT} (n=21)**	***NPM1*^{WT} *FLT3*^{ITD} (n=7)**	***NPM1*^{MUT} *FLT3*^{ITD} (n=16)**	**P***
Status after course 1											
CR (%)	20 (56)	32 (86)		36 (72)	16 (70)		18 (62)	18 (86)	2 (29)	14 (88)	0.009
RD (%)	9 (25)	2 (5)		6 (12)	5 (22)		5 (17)	1 (5)	4 (57)	1 (6)	
ID (%)	7 (19)	3 (8)	0.005	8 (16)	2 (8)	0.99	6 (21)	2 (10)	1 (14)	1 (6)	
CR ever	24 (67)	33(89)	0.03	38 (76)	19 (83)	0.8	19 (66)	19 (90)	5 (71)	14 (88)	0.1

*P values refer to attaining CR vs not attaining CR.

Abbreviations: CR, complete remission; CR ever, reflects patients who attain CR at some point regardless of the number of treatment courses; ID, induction death; RD, resistant disease.

analysis. All four factors maintained their significant association with the achievement of CR (WCC, P=0.002; *NPM1* status, P<0.0001; cytogenetic risk group, P=0.002; age P=0.008). *FLT3*^{ITD} status remained non-significant for predicting response to induction treatment (P=0.3).

4.3.6 Overall survival and disease-free survival

The major outcomes of OS and DFS were then assessed for the intensively treated patient cohort (including APL) (n=155). The median OS for the whole cohort was 815 days, with 3-year and 5-year actuarial OS of 47% and 39% respectively. For the 116 patients achieving remission, the median DFS was 852 days, with 3-year and 5-year actuarial DFS of 47% and 43% respectively.

4.3.6.1 Overall survival and disease free survival according to age and cytogenetics

The outcomes of the intensively treated cohort for DFS and OS according to age are shown in Table 4.18 and Figure 4.7. Age ≥60 years was associated with a significantly decreased 5-year DFS and OS. There was also a significant difference in DFS and OS according to cytogenetic risk group. In keeping with the MRC risk groups, patients in the favourable cytogenetic group had the best DFS and OS, and patients with adverse cytogenetics the worst (Table 4.18, Figure 4.8). Similar results were seen when only patients aged <60 years (an age group that more closely resembles the MRC study) were assessed, with 5-year OS in the favourable, intermediate, and adverse cytogenetic groups of 86%, 52%, and 8% respectively (P<0.0001).

Again, in keeping with the MRC data, within the favourable risk-risk group, patients with APL performed better than those with either t(8;21) or inv(16)/t(16;16), although the numbers in each subgroup were small and this did not reach statistical significance, with 5-year OS of 100%, 83%, and 50% respectively (P=0.16). Previous studies have shown an increased relapse risk in patients with inv(16)/t(16;16) compared to t(8;21), but because of their ability to be salvaged, they

Table. 4.18 Outcome in the 155 intensively treated patients according to age and cytogenetic risk group

	No. pts (%)	Median DFS (days)	DFS@5 years (%)	P HR (95% CI)	Median OS (days)	OS@5 years (%)	P HR (95% CI)
Age (%)				0.02 0.44 (0.24-0.81)			<0.0001, 0.31 (0.19-0.51)
<60 years	102 (66)	2238	52%		2287	50%	
≥60 years	53 (34)	392	19%		282	19%	
Cytogenetics (% all/known)				0.0007			<0.0001
Favourable	28 (18/21)	Undefined	76%		Undefined	83%	
Intermediate	80 (52/61)	764	40%		559	38%	
Adverse	23 (15/18)	392	27%		210	7%	
Failed/UK	24 (15/-)	414.5	14%		335.5	27%	

Abbreviations: CI, confidence intervals; DFS, disease-free survival; HR, hazard ratio; OS, overall survival

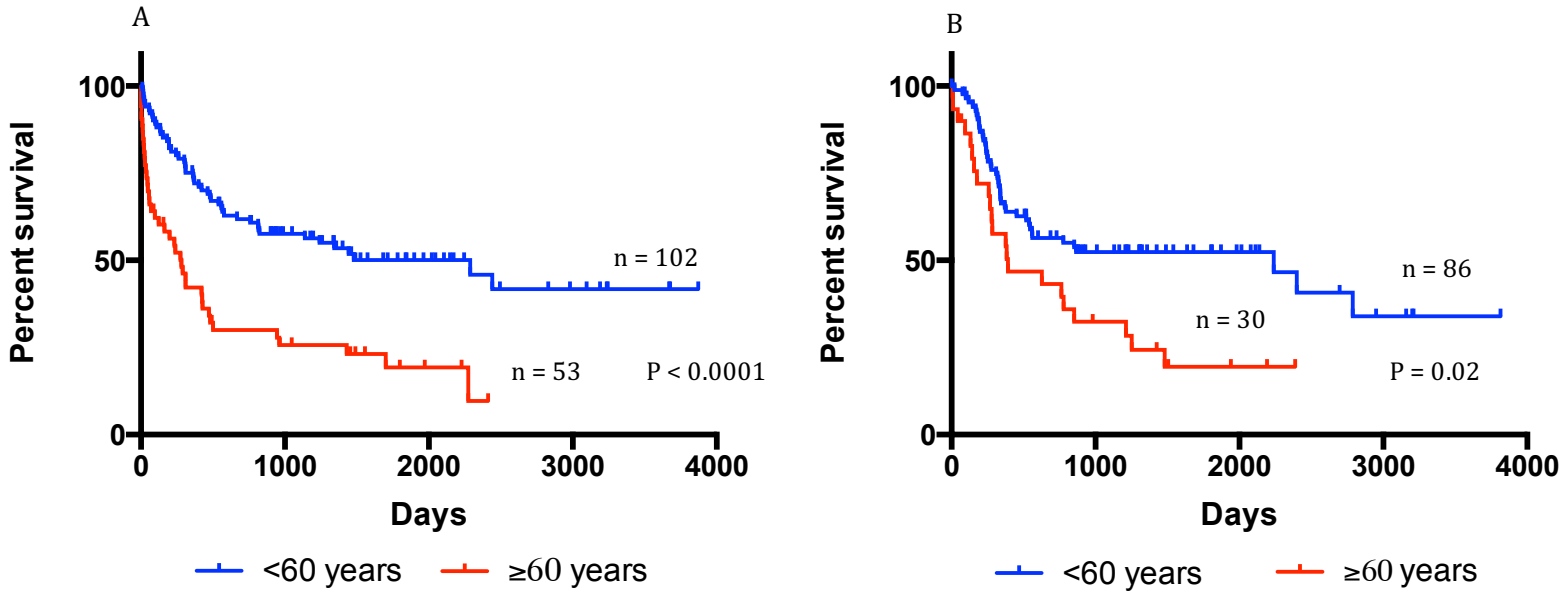


Figure 4.7 Outcome according to age in the total group of 155 intensively treated patients. (A) Overall survival and (B) disease-free survival.

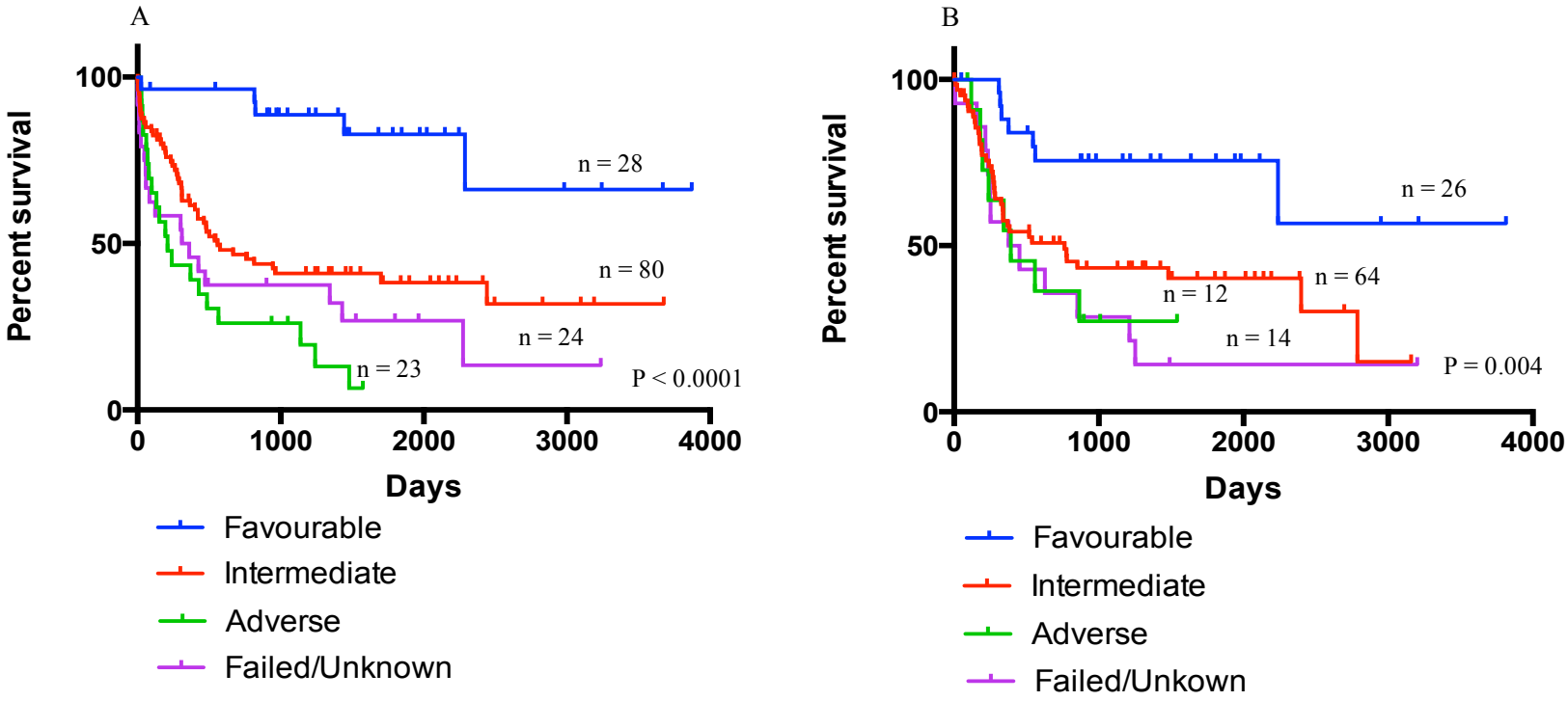


Figure 4.8 Outcome according to MRC cytogenetic risk group in the total group of 155 intensively treated patients. (A) Overall survival and (B) disease-free survival.

have comparable OS (Allen *et al*, 2013). In keeping with this, there was a significant difference in DFS between the groups, with 5-year DFS for APL, t(8;21) and inv(16)/t(16;16) 100%, 67%, and 20% respectively (P=0.002).

4.3.6.2 Survival analysis according to genotype

The DFS and OS in 73 intensively treated patients with intermediate-risk cytogenetics according to the *NPM1*^{MUT} and *FLT3*^{ITD} status are shown in Table 4.19. Patients with *NPM*^{MUT} had significantly improved DFS and OS compared to *NPM1*^{WT} patients (Figure 4.9). There was no association between *FLT3*^{ITD} status and either DFS or OS (Figure 4.10). There was a trend for inferior survival when patients <60 years were analysed separately for *FLT3*^{ITD} and *FLT3*^{WT}, with 5-year DFS 39% vs 58% (HR 1.94, 95% CI 0.76 - 4.94, P=0.16) and 5-year OS 43% vs 56% (HR 1.47, 95% CI 0.62-3.52, P=0.76).

4.3.6.2.1 Intermediate-risk patients according to combined *NPM1*/*FLT3* genotype

For the cohort of intensively treated intermediate-risk patients with known *NPM1* and *FLT3* status (n=73), DFS and OS were assessed according to the combined *NPM1* and *FLT3* status. The median OS for the 4 genotype groups, *NPM1*^{MUT}*FLT3*^{ITD}, *NPM1*^{MUT}*FLT3*^{WT}, *NPM1*^{WT}*FLT3*^{ITD}, and *NPM1*^{WT}*FLT3*^{WT}, was 815 days, undefined, 309 days, and 366 days respectively (P=0.0004) and the 5-year OS was 49%, 73%, 0% and 16% respectively (Table 4.19, Figure 4.11A). The median DFS for the 4 genotypes was 777 days, undefined, 220 days, and 325 days respectively (P=0.0003, and the 5-year DFS 49%, 70%, 0% and 19% respectively (Table 4.19, Figure 4.11B). When only those patients <60 years were considered, the 5-year DFS was 53%, 61%, 0%, and 30% respectively (P=0.01), and the 5-year OS was 53%, 87%, 0%, and 23% (P=0.008).

Thus the *NPM1*^{MUT} patients performed as expected, with improved DFS and OS when considered in isolation, and they performed particularly well in the absence of

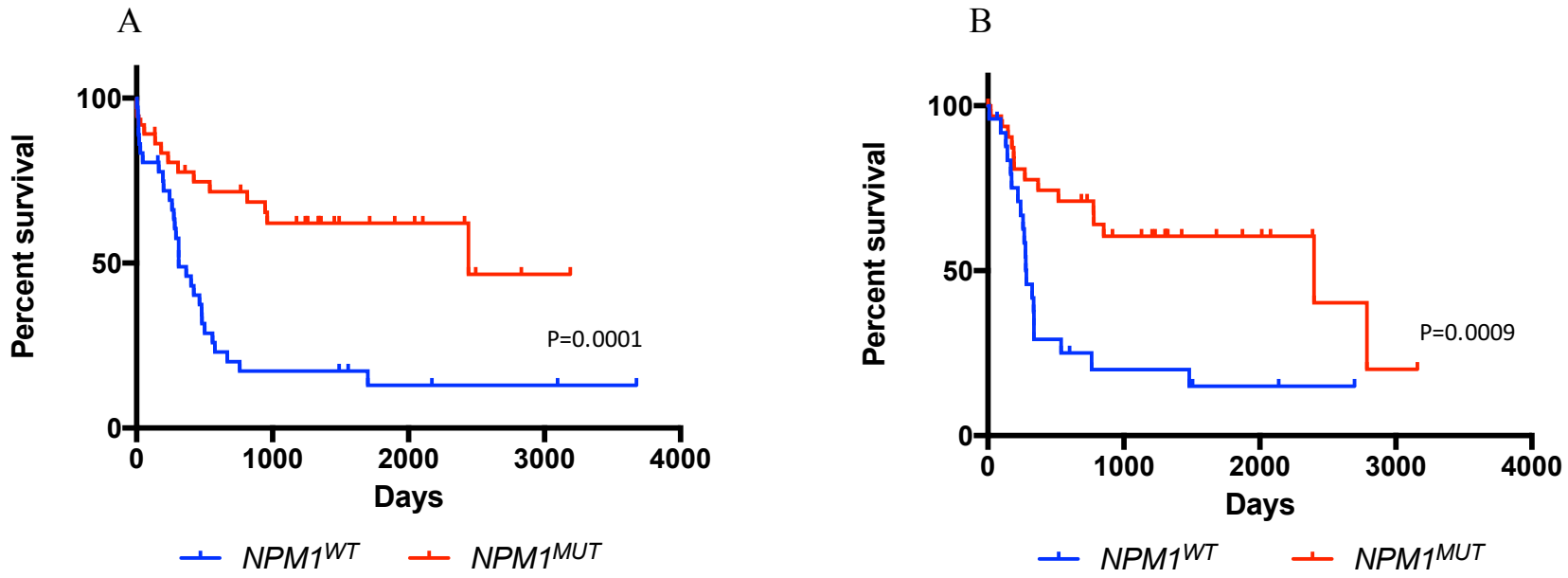


Figure 4.9 Outcome according to *NPM1* mutation status in the 73 intensively treated patients with intermediate-risk cytogenetics and known mutation status. (A) Overall survival and (B) disease free-survival.

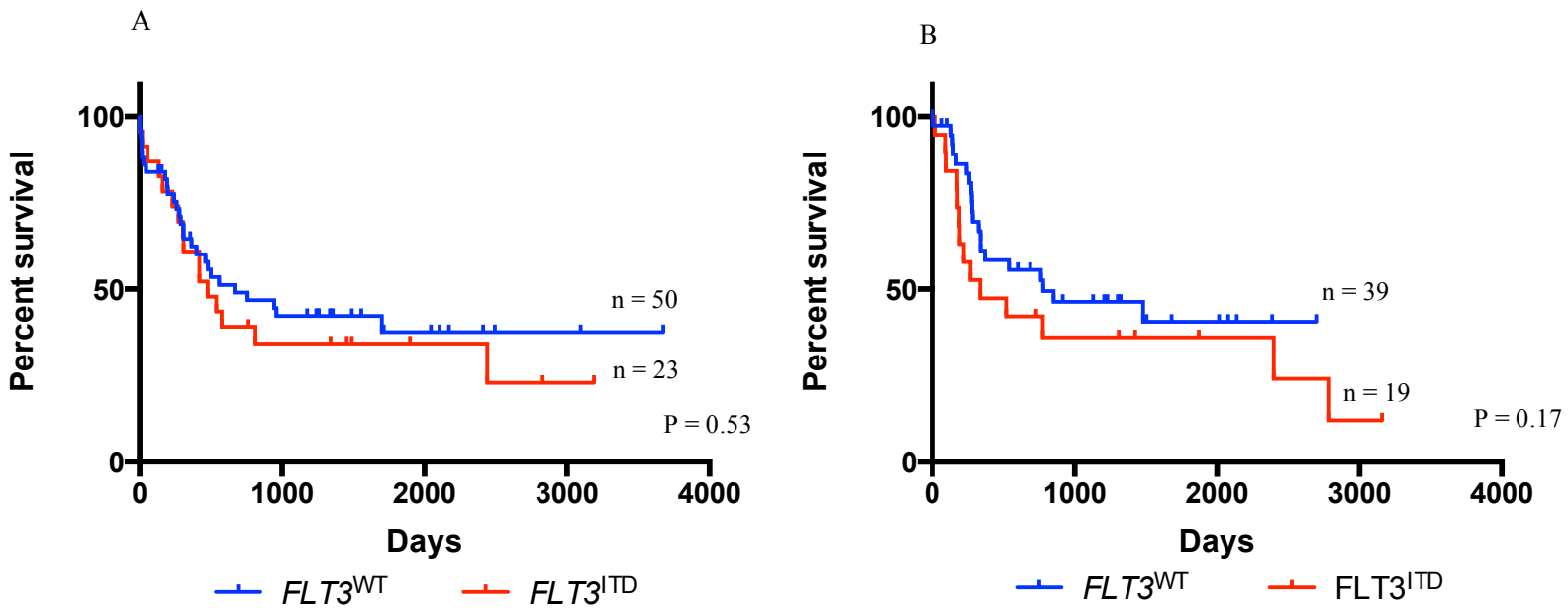


Figure 4.10 Outcome according to *FLT3* mutation status in the 73 intensively treated patients with intermediate-risk cytogenetics and known mutation status. (A) Overall survival and (B) disease-free survival.

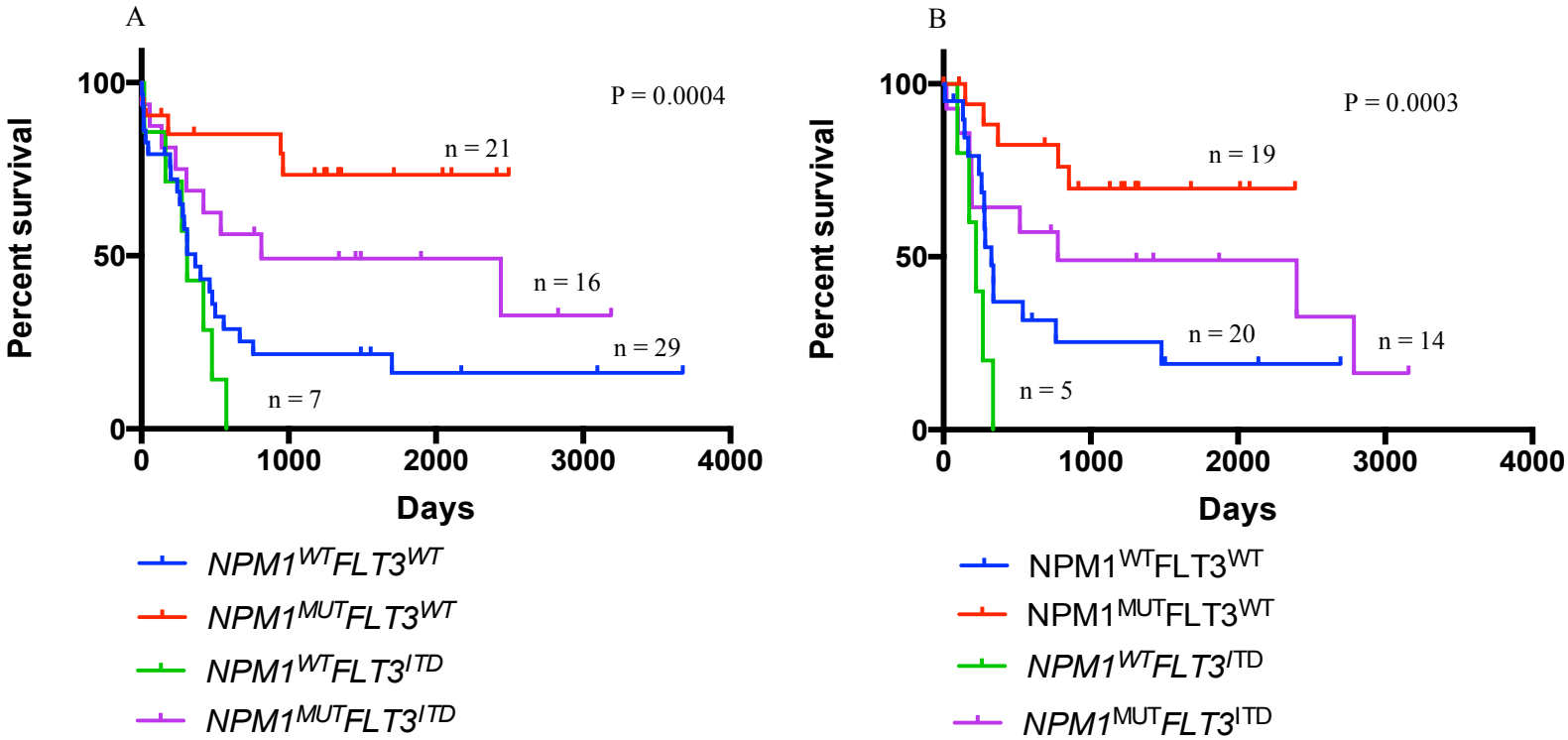


Figure 4.11 Outcome according to the combined *NPM1/FLT3* mutation status in the 73 intensively treated patients with intermediate-risk cytogenetics and known mutation status. (A) Overall survival and (B) disease-free survival.

Table 4.19 Outcome according *NPM1* and FLT3 mutation status in 73 intensively treated patients with intermediate-risk cytogenetics

	No. pts (%)	Median DFS (days)	DFS@5 years (%)	P HR (95% CI)	Median OS (days)	OS@5 years (%)	P HR (95% CI)	CIR@5 years (%)	P
***NPM1* status**				0.0009 0.29 (0.14-0.60)			0.0001 0.30 (0.16-0.55)		0.001
NPM1^{MUT}	37 (51)	2399	60%		2440	62%		30%	
NPM1^{WT}	36 (49)	283	15%		311	13%		73%	
***FLT3* status**				0.29 1.50 (0.71-3.17)			0.9 1.23 (0.65-2.32)		0.87
FLT3^{ITD}	23 (32)	336	36%		478	34%		47%	
FLT3^{WT}	50 (68)	778	41%		668	38%		50%	
Combined				0.0003			0.0004		0.002
NPM1^{MUT}*FLT3*^{ITD}	16 (22)	777	49%		815	49%		35%	
NPM1^{MUT}*FLT3*^{WT}	21 (29)	Undefined	70%		Undefined	73%		24%	
NPM1^{WT}*FLT3*^{ITD}	7 (10)	220	0%		309	0%		80%	
NPM1^{WT}*FLT3*^{WT}	29 (40)	325	19%		366	16%		71%	

Abbreviations: CI, confidence intervals; CIR, cumulative incidence of relapse; DFS, disease-free survival; HR, hazard ratio; OS, overall survival

a *FLT3*^{ITD} (Table 4.19). In contrast, *FLT3*^{ITD} when considered in isolation was not associated with an inferior OS. Although some of the studies investigating *FLT3*^{ITD} without incorporating *NPM1* status have demonstrated an inferior survival (Kottaridis *et al*, 2001), some large studies, including the largest and third largest studies of adults (Schnittger *et al*, 2002; Thiede *et al*, 2002) also failed to see an impact prognostic impact of *FLT3*^{ITD}. Furthermore, the finding of inferior survival for *NPM1*^{MUT}*FLT3*^{ITD} genotype patients was in keeping with the major studies considering the combination of mutations in both unselected cohorts and in intermediate karyotype/NK cohorts (Table 4.3). One anomaly of the UCLH cohort compared to published data was the apparent inferior outcome of *NPM1*^{WT}*FLT3*^{WT} compared to *NPM1*^{MUT}*FLT3*^{ITD} patients for both DFS and OS, although this did not reach statistical significance. When these 2 groups were compared directly, the 5-year DFS was 49% vs 19% (HR 1.96, 95% CI 0.94 - 3.79, P=0.08) and 5-year OS 49% vs 16% (HR 1.69, 95% CI 0.76 - 3.75, P=0.2).

4.3.6.2.2 Outcome according to *FLT3*^{ITD} burden

The outcome was then assessed according to *FLT3*^{ITD} burden to see if it worsens with increasing mutation level, as has been suggested (Gale *et al*, 2008; Pratcorona *et al*, 2013), and whether those patients with the lowest mutant burden still have the inferior outcomes seen in the *FLT3*^{ITD} group as a whole when compared to *FLT3*^{WT}. This is a contentious issue as some studies have shown low-level *FLT3*^{ITD} have the same outcome as *FLT3*^{WT}, while other larger studies have shown that OS and CIR are similar for *FLT3*^{ITD} levels of <25% and 25-50% (Linch *et al*, 2014). Although there was a shift in OS rates for *FLT3*^{ITD} patients with <25% mutant if an attempt was made to adjust for sample purity, this did not affect the CIR (Linch *et al,* 2014).

As before, three groups were defined: *FLT3*$^{ITD\text{-}LOW}$ (<25% mutant), *FLT3*$^{ITD\text{-}INT}$ (25-50%), and *FLT3*$^{ITD\text{-}HIGH}$ (>50%). In the intermediate cytogenetic risk group, for intensively treated patients, the median OS for the 3 groups was 422, 2440, and 233 days respectively (P=0.003). The 3-year OS was 20%, 53%, and 0% (Figure 4.12A). The median DFS for the 3 groups was 181, 2399, and 175 days (P=0.0002) and the

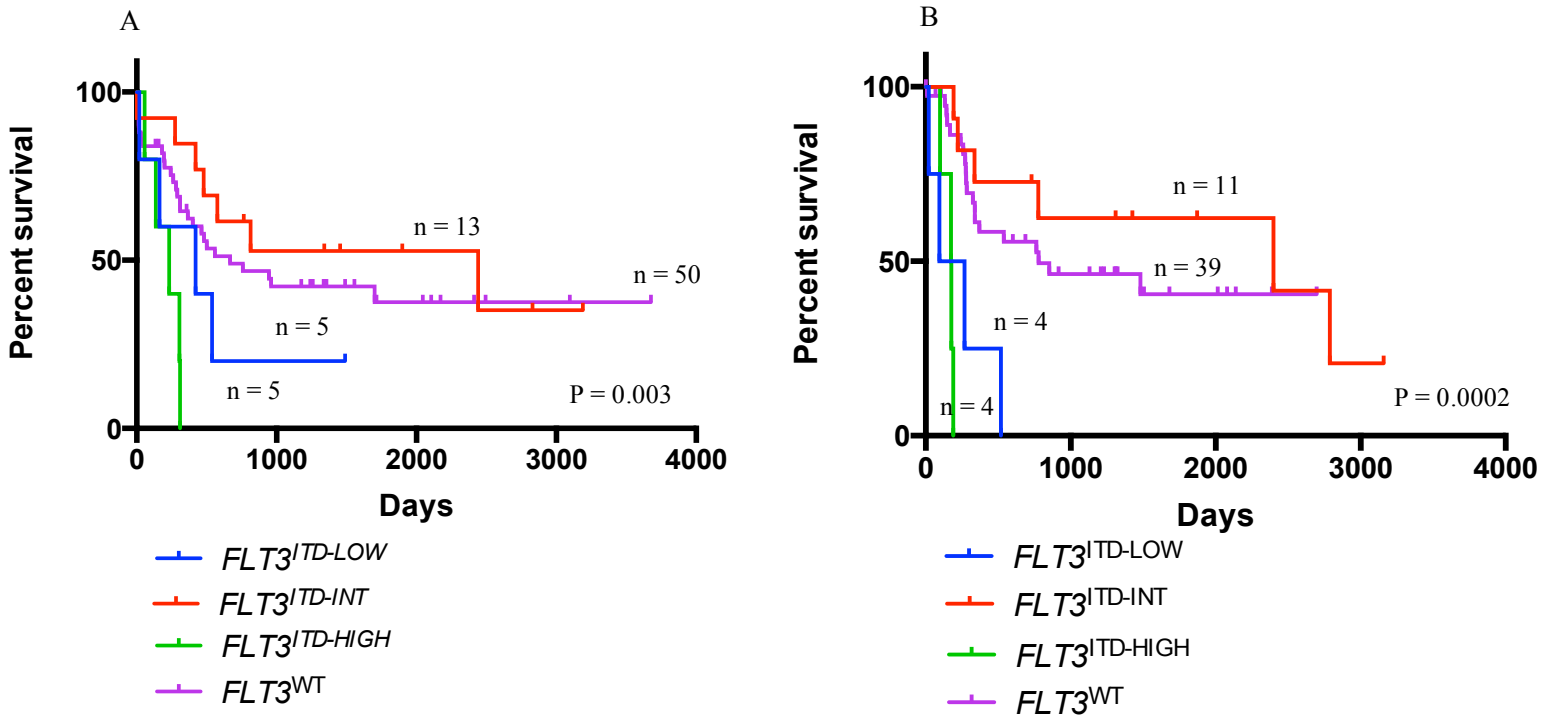


Figure 4.12 Outcome according to *FLT3*^{ITD} mutant level in 23 intensively treated patients with intermediate-risk cytogenetics. (A) Overall survival and (B) disease-free survival. *FLT3*$^{ITD-LOW}$ (<25% mutant), *FLT3*$^{ITD-INT}$ (25-50% mutant), *FLT3*$^{ITD-HIGH}$ (>50% mutant). *FLT3*^{WT} is included for comparison. P values refers to analysis with *FLT3*^{WT} excluded. For (A) P = 0.009 and (B) P<0.0001 when *FLT3*^{WT} is included.

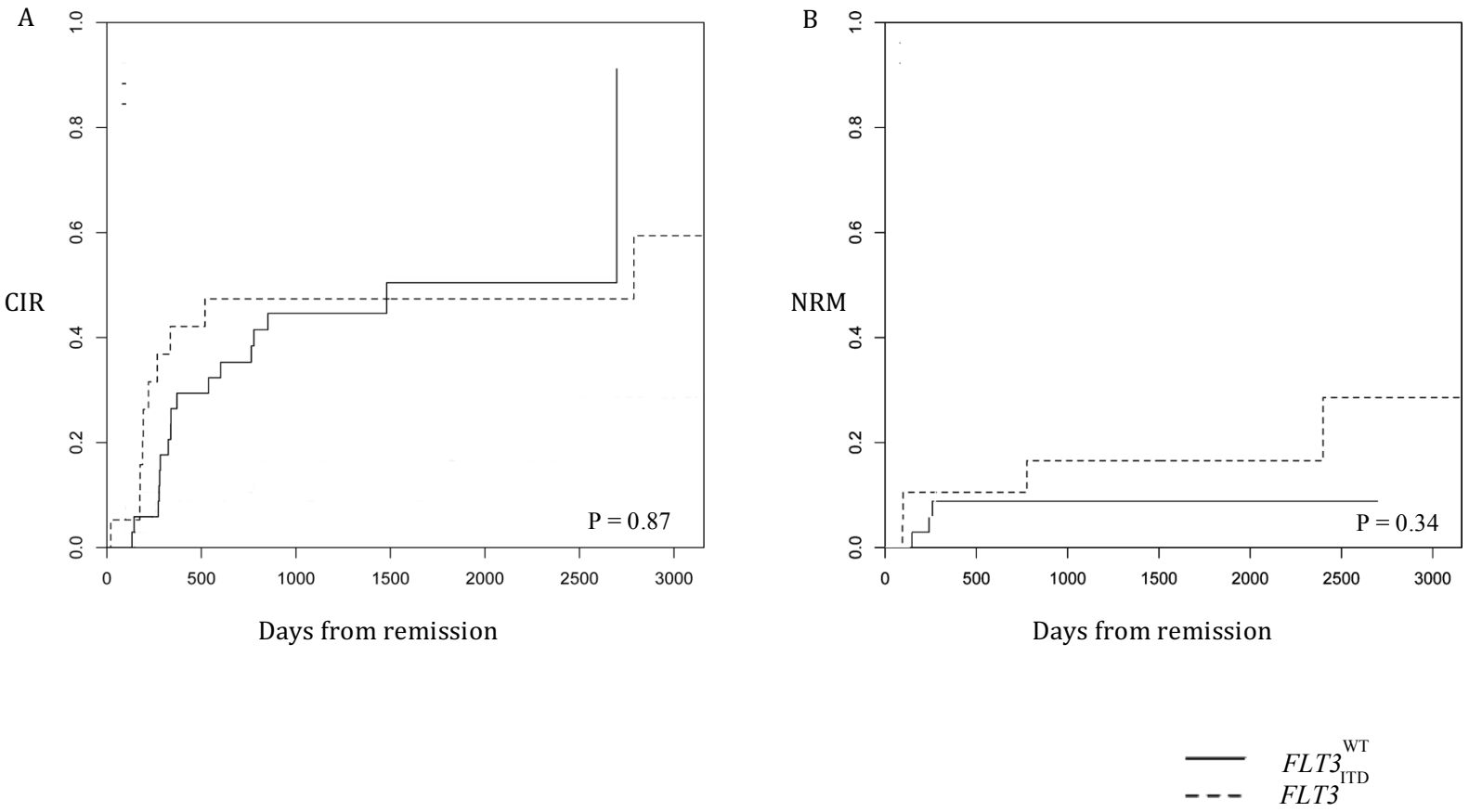


Figure 4.13 Outcome following remission in intensively treated patients with intermediate-risk cytogenetics according to *FLT3* mutation status. (A) Cumulative incidence of relapse (CIR) and (B) non-relapse mortality (NRM).

3-year DFS 0%, 62%, and 0% respectively (Figure 4.12B). Thus, as with the *FLT3*^{ITD} group as a whole, the largest group of patients with *FLT3*$^{ITD\text{-}INT}$ had a similar outcome to *FLT3*^{WT} patients. In contrast, patients with either *FLT3*$^{ITD\text{-}LOW}$ or *FLT3*$^{ITD\text{-}HIGH}$ had a poorer outcome with actuarial survival of 0% at 3 years, albeit with small numbers in these groups. Previous reports from MRC studies have shown that, unless adjusting for sample impurity, *FLT3*$^{ITD\text{-}LOW}$ patients have poorer outcome than *FLT3*^{WT} and a CIR comparable to higher *FLT3*^{ITD} levels (Gale *et al*, 2008; Linch *et al*, 2014).

4.3.7 Relapse-risk in the intermediate-risk cytogenetic group according to genotype

The CIR was then assessed in the intermediate-risk cytogenetic group in order to assess whether these differences in OS and DFS according to genotype could be attributed to differences in the risk of relapse. As a large number of patients in the intermediate-risk group, particularly those with higher risk genotypes, undergo HSCT, a competing risk methods was employed to distinguish between those patients who relapsed as compared to those patients who died in remission, predominantly for treatment-related causes, e.g. infection, or graft-versus-host disease (GVHD), so called non-relapse mortality (NRM).

A total of 57 patients with intermediate-risk cytogenetics and with *NPM1* and *FLT3* status available attained remission and were eligible for the competing risks analysis. In the assessment according to *FLT3*, the 5-year CIR was 50% in the *FLT3*^{WT} group versus 47% in the *FLT3*^{ITD} group (P=0.87) (Table 4.19, Figure 4.13A). There was also no significant difference in the NRM at 5 years (9% vs 17%, P=0.34) (Figure 4.13B). In the same 57 patients, a competing risks analysis according to *NPM1* mutation status showed that there was no difference in the NRM rate between patients who were *NPM1*^{WT} and those who were *NPM1*^{MUT} (14% vs 10%, P=0.97) (Figure 4.14B). However, the *NPM1*^{WT} patients had a significantly increased CIR at 5 years compared to the *NPM1*^{MUT} patients (73% vs 30%, P=0.001) (Table 4.19, Figure 4.14A).

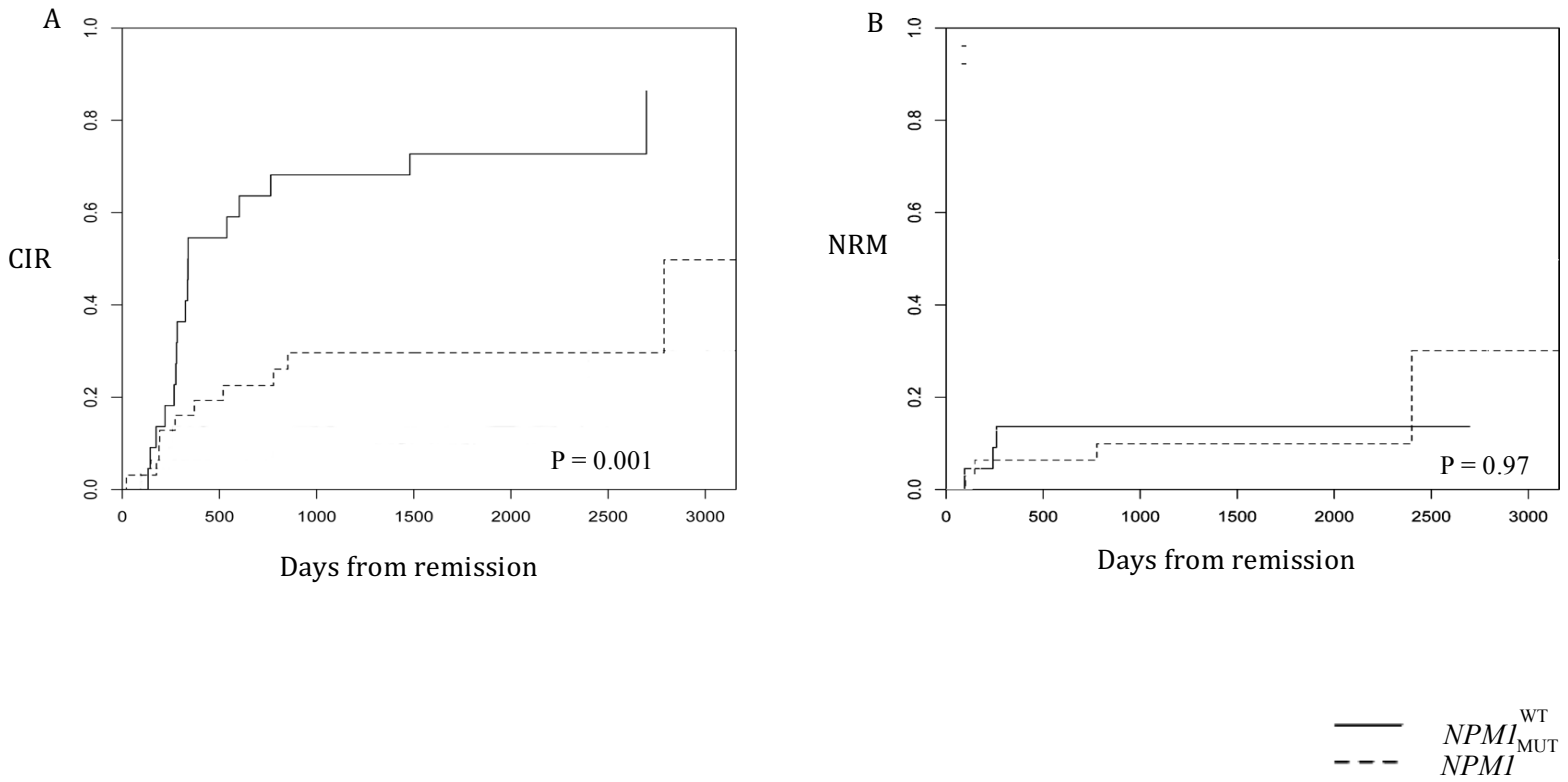


Figure 4.14 Outcome following remission in intensively treated patients with intermediate-risk cytogenetics according to *NPM1* mutation status. (A) Cumulative incidence of relapse (CIR) and (B) non-relapse mortality (NRM).

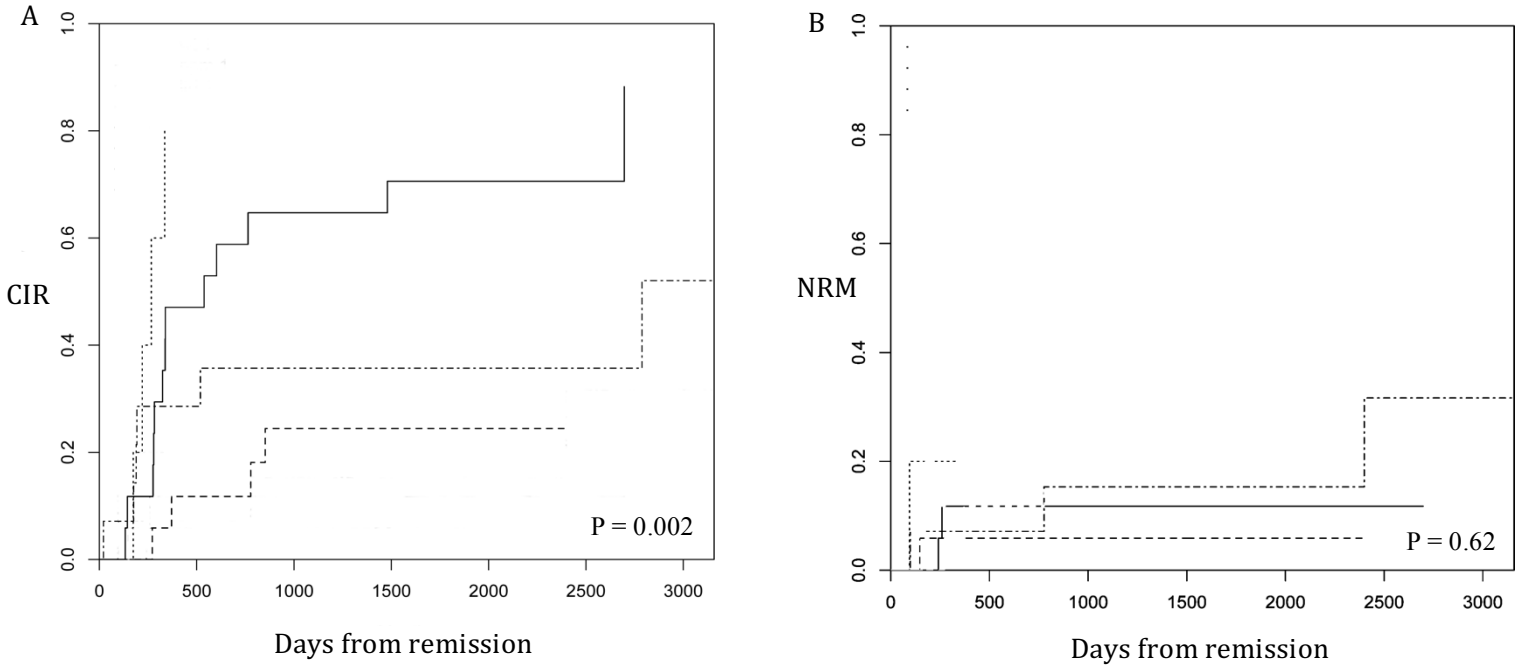


Figure 4.15 Outcome following remission in intensively treated patients with intermediate-risk cytogenetics according to the combined *NPM1/FLT3* mutation status. (A) Cumulative incidence of relapse (CIR) and (B) non-relapse mortality.

The CIR and NRM were then assessed according to the combined *NPM1/FLT3* genotypes (Table 4.19, Figure 4.15). The 5-year CIR for the $NPM1^{MUT}FLT3^{ITD}$, $NPM1^{MUT}FLT3^{ITD}$, $NPM1^{WT}FLT3^{ITD}$, and $NPM1^{WT}FLT3^{WT}$ groups were 35%, 24%, 80%, and 71% respectively (P=0.002), and there was no difference in the NRM (15%, 6%, 20%, 12%; P=0.62). When the $NPM1^{MUT}FLT3^{ITD}$ and $NPM1^{WT}FLT3^{WT}$ patients were compared directly, the difference in CIR was not statistically significant (P=0.08). The difference in relapse risk between the groups was not obviously due to a large difference in the rate of HSCT between the groups as the proportion of patients undergoing HSCT in the respective groups was 29%, 26%, 40%, and 32% respectively.

Thus, the commonly reported decreased relapse risk associated with $NPM1^{MUT}$ was seen most prominently in the context of a $FLT3^{WT}$ genotype, however, in the context of a $FLT3^{ITD}$ it did appear to mitigate the possible inferior outcome. The inferior CIR reported as the reason for inferior OS in $FLT3^{ITD}$ patients was not seen in the group as a whole, but was seen in the context of $NPM1^{WT}$.

4.4 Discussion

This chapter gives details of a cohort of 181 in-house patients with a new diagnosis of AML that were selected because they had diagnostic trephine biopsies available for the assessment of DNA replication licensing proteins and cell cycle status using IHC. It is noteworthy that the number of patients with available biopsies in the first 2 years of the study (2003 and 2004) is fewer than in later years (2005 to 2011). There are a number of possible reasons for this apparent increase with time. Firstly, it may represent a true increase in the number of patients either being diagnosed with AML or, more likely, being referred to UCLH for intensive treatment. Secondly, the ease of identifying appropriate cases from electronic and paper records diminished on searching further back into the archives, although the number of sources used to identify patients, including pathology and haematology laboratory records, should have lessened the likelihood of having missed cases.

Referral patterns for intensive treatment may also explain why this cohort is younger (median age 56 years) than cohorts based on trials or populations, where the median age of diagnosis is often ≥65 years. However, UCLH is a central referring hospital for intensive treatment, which may skew the AML population towards younger patients deemed fit for intensive induction therapy, with or without HSCT, and away from older patients receiving non-intensive treatment or supportive therapy. Furthermore, because older patients are more likely to have secondary disease, it may be that a biopsy sample is deemed unnecessary for a diagnosis of AML where either PB or BMA may be sufficient for diagnostic purposes.

Despite these caveats on age, there were a number of features of the cohort that it made it potentially useful for a study. Firstly, the younger cohort could be readily compared with large clinical trials such as the MRC/NCRI AML trials, which have been extensively described in terms of cytogenetic and molecular features, particularly in the younger-aged patient cohorts. Two of the main studies describing MRC patient data that were used as comparators in this chapter focussed on younger adults. The median age of patients in the cytogenetic study (Grimwade *et al*, 2010) was 44 years and in the study of *NPM1* and *FLT3* it was 43 years (Gale *et al*, 2008). Despite this disparity in overall age, the UCLH cohort performed generally well in terms of relative frequency of cytogenetic groups and *NPM1/FLT3* genotype. The only apparent discordance was the increased frequency of patients with adverse cytogenetics seen in the cohort compared to the MRC data, even when only patients <60 years were considered. The cohort as a whole had overall frequencies of $NPM1^{MUT}$, $FLT3^{ITD}$, and combined genotypes in keeping with larger cohorts including the MRC studies, particularly when the effect of the large numbers of patients with adverse cytogenetics was mitigated by only considering the relative frequencies in the intermediate-risk cytogenetics and NK groups. Furthermore, the expected association between $NPM1^{MUT}$ and $FLT3^{ITD}$ genotypes was observed, as well as the association for both mutations with intermediate-risk cytogenetics. The data on $NPM1^{MUT}$ and $FLT3^{ITD}$ mutant burden were also consistent with large trials, including MRC data (Gale *et al*, 2008). Whereas there was a large spread of $FLT3^{ITD}$ mutant levels between patients, with 58% of patients having levels of 25-50%, the $NPM1^{MUT}$ burden was tightly grouped with 92% in the 25-50% mutant range, indicating heterozygous mutation in all or almost all leukaemic cells. Other features consistent with published cohorts were the association of both

NPM1^{MUT} and *FLT3*^{ITD} with increased WCC, and the further association between increasing WCC with increasing *FLT3*^{ITD} burden.

When considering FAB types, the negative association between *FLT3*^{ITD} and M2 noted by others was seen, but not the association with M5. Furthermore, *NPM1*^{MUT} was associated with M5 but not M4 subtypes. This may reflect the difficulty in trying to retrospectively infer FAB type from historic laboratory reports, or inaccuracy of reporting given the move away from using FAB type and the increasing reliance on cytogenetic and molecular data rather than morphologic and cytochemical data. In terms of assessing the UCLH patients however, these are relatively minor points in a cohort that performs very well in terms of demographic equivalence to larger cohorts, in particular those from the MRC UK trials.

In terms of outcome, particularly response to induction therapy, it is noteworthy that the majority of patients in the cohort who were treated intensively received DA50 or ADE, two treatments with essentially similar outcomes that have formed the backbone of MRC induction therapy. This was important when comparing outcomes of treatment with other trials, including the UK-based studies. However, when considering IDs, the largest study reported is of over 3000 patients treated on unspecified protocols at institutions in the USA (Walter *et al*, 2011). The authors found that the two most important factors predicting for death in the first four weeks of treatment were performance status and age, with higher WCC at diagnosis also predicting for early NRM. In the UCLH cohort, increasing age was also associated with an increased risk of early death. The importance of WCC and age was also demonstrated in multivariate analysis for response to intensive induction therapy. However, the performance status, a key predictor of response to treatment and survival, was not reliably available for this UCLH cohort. Patients entering clinical trials routinely have an ECOG performance status recorded electronically at the point of trial entry or registration. However, this information is often not available in the hospital record, or patients are being treated off-trial (72 of 175, 41% in this study), and trying to retrospectively deduce the performance status from the patient's notes was very difficult and felt to be too unreliable to be of value or to allow accurate analysis.

When considering DFS and OS, the importance of patient age for outcome was evident. For intensively treated patients, OS was markedly lower in the older patients (≥60 years). Nonetheless, the median age for the whole cohort was 56 years, the majority of patients were aged <60 years, the majority of those ≥60 years were treated intensively, and the prognostic factors seen in the outcome data were essentially the same, whether the whole intensively treated cohort or only those patients <60 years were being considered.

The outcome data for the cytogenetic risk groups that has been established using over five and a half thousand patients (Grimwade *et al*, 2010) also held true in this smaller UCLH cohort. Patients with APL had a superior outcome to all other forms of AML, and there were clear survival differences for patients within the favourable, intermediate, and adverse groups (Figure 4.8A). Interestingly, even within the favourable group, the greater propensity for patients with inv(16)/t(16;16) to relapse compared t(8;21) as evidenced by a markedly inferior DFS but similar OS was as previously noted in larger studies of the CBF-leukaemias treated on MRC trials (Allen *et al*, 2013). The patients with adverse-risk cytogenetics performed as poorly as in other studies.

For outcome according to genotype, the impact of both mutations was generally as expected according to the larger published cohorts in that the presence of $NPM1^{MUT}$ predicted for achievement of CR in both univariate and multivariate analysis whereas the presence of $FLT3^{ITD}$ did not. Furthermore, patients with $NPM1^{MUT}$ had a clear survival advantage for both OS and DFS. Patients with $FLT3^{ITD}$ did not have clearly inferior OS and DFS when ITDs were considered in isolation. However, when the *NPM1* status was also taken into consideration, patients who had a $NPM1^{WT}FLT3^{ITD}$ genotype had a significantly inferior OS and DFS. A study of patients treated on MRC trials published prior to the discovery of $NPM1^{MUT}$ showed a clearly inferior OS for patients with $FLT3^{ITD}$ (Kottaridis *et al*, 2001). However, similarly to this UCLH cohort, this was not evident in some other large studies (Schnittger *et al*, 2002; Thiede *et al*, 2002). Furthermore, when the combined *NPM1/FLT3* genotype was taken into consideration, the UCLH cohort performed similarly to the large study of MRC trial patients (Gale *et al*, 2008) in that the best outcomes were seen in $NPM1^{MUT}FLT3^{WT}$ patients and the worst outcomes in the group with $NPM1^{WT}FLT3^{ITD}$.

When outcome was assessed according to *FLT3*^{ITD} burden, patients in the intermediate-risk cytogenetic group and with the highest mutant burden (>50% mutant), representing patients with at least some leukaemic cells with homozygous mutations, had inferior outcome to those patients with intermediate mutant burden (25-50%). However, patients with the lowest mutant burden (<25%) had similar outcomes to those patients with the highest mutant burden. *NPM1*^{MUT} were found at the expected frequency within the *FLT3*$^{ITD\text{-}LOW}$ group (40% in this study) compared with the large study of patients treated on MRC trials (Gale *et al*, 2008) (51% of the cohort). Importantly, this study reflected the findings of a recent report of MRC trial patients (Linch *et al*, 2014), in which, if no adjustment was made for sample purity, *FLT3*$^{ITD\text{-}LOW}$ patients had similar OS to *FLT3*^{WT}, and even if adjustment was made for sample purity, they had similar CIR. This was independent of *NPM1* mutation status. This is important as two prior smaller studies suggest that in *NPM1*^{MUT} patients, only those with *FLT3*^{ITD} mutant level ≥33% (i.e. a WT:mutant allele ratio of >0.5) had a worse outcome (Schnittger *et al*, 2011; Pratcorona *et al*, 2013). In any case, in this UCLH cohort, only 8 patients had a *FLT3*^{ITD} mutant level <33%, of whom only 5 had *NPM1*^{MUT}, and this is too small a number of patients from which to draw firm conclusions on subgroup analysis.

As *NPM1*^{MUT} had improved CR rates, it was next assessed whether the improved survival was also because of decreased relapse rates. In addition, many patients with *FLT3*^{ITD} receive HSCT, and inferior survival may be due to either relapse or NRM. A competing risks analysis was therefore performed, with NRM and relapse as mutually exclusive events. This showed that patients with *NPM1*^{MUT} have a significantly decreased CIR, and those with *FLT3*^{ITD} mutations were only associated with an increased CIR in the absence of *NPM1*^{MUT}. There were no differences in NRM between the groups. This lack of increased CIR with *FLT3*^{ITD} was at odds with data from the MRC10, 12, and 15 trial patients (Kottaridis *et al*, 2001; Gale *et al*, 2008; Linch *et al*, 2014), as was the trend towards an increased CIR in the *NPM1*^{WT}*FLT3*^{WT} cohort. However, the significant decrease in CIR in *NPM1*^{MUT} patients, as well as the highest CIR in the *NPM1*^{WT}*FLT3*^{ITD} group, was in keeping with these studies.

There are several limitations to the present cohort. Firstly, it is small with a wide age range. The project started in 2011, with all samples collected retrospectively. Although

taking more recent cases than 2011 would have been feasible, there would only have been limited follow-up time available. Such cases may have been useful for response to induction therapy, but the mean time to relapse for patients with *NPM1*^{MUT} was 616 days, which would suggest that a minimum follow-up time of 2 years would be necessary. An attempt to find cases earlier than 2003 was also problematic due to difficulties in getting accurate identification from records, lack of availability of electronic records, and challenges in locating archived tissue. The number of patients in these earlier years was also limited, potentially for the reasons outlined above. A further way of expanding numbers would have been to use patients from other large teaching hospitals. However, the practice of performing a biopsy in parallel with each diagnostic aspirate is not universal, and it is not a requirement in guidelines where adequate diagnostic material is available from aspirate and blood and the diagnosis is not in doubt (British Committee for Standards in *et al*, 2006). After contacting three large UK institutions failed to identify further cases, this approach was not pursued.

A further potential limitation for evaluating outcomes of the cohort was the lack of uniformity of treatment, a result of the large numbers of patients recruited to national clinical trials that included several different randomisations. However, it should be noted that where variations in treatment in such trials have influenced outcome, the benefits of the different treatments have been modest and only seen using hundreds of patients. Indeed, the outcome data for large cohorts demonstrating the importance of cytogenetics, *NPM1* and *FLT3* status have usually merged the data of patients receiving different forms of intensive therapy, and many include patients from different trials. Furthermore, with the exception of some of the earlier studies reporting the impact of *FLT3*^{ITD} on outcome, the prognostic impact of cytogenetics, *NPM1*^{MUT}, and the *NPM1*/*FLT3* genotype are broadly similar between reports from groups using different treatment regimens.

Only limited genetic profiling of the cohort was performed, *NPM1*^{MUT} and *FLT3*^{ITD} being the most frequent and prognostically important mutations, albeit in cytogenetically intermediate-risk patients. However, as outlined in chapter 1, many other recurrent mutations with varying degrees of prognostic importance have recently been described. Some of these are hotspot mutations that can be rapidly identified (e.g. *IDH1* and *IDH2*), others such as mutations in *DNMT3A* require analysis of a greater

number of exons, and for some patients there would have been insufficient amounts of DNA to allow for such analysis. As yet, they are not part of the ELN classification, and for some their impact remains controversial.

In summary, this chapter has defined a medium-sized heterogeneous cohort of patients with AML with diagnostic biopsies available for IHC analysis of DNA replication licensing proteins and cell cycle analysis. Importantly, the main prognostic demographic features of AML were found to be present at equivalent rates and generally associated with the same clinical features and survival outcomes reported in the literature for studies of larger patient cohorts.

CHAPTER 5. ANALYSIS OF DNA REPLICATION LICENSING PROTEINS AND CELL CYCLE STATUS IN PATIENTS WITH AML

5.1 Introduction

In chapter 1, the biology of the normal G_1-S phase transition was described. Using this knowledge of biology, it has been shown that stages of the cell cycle can be defined in fixed samples by the detection of DNA replication licensing proteins using IHC. Furthermore, it was shown in chapter 3, that in the case of patients with AML, IHC on fixed biopsies provides a more accurate assessment of the *in vivo* state than does assessment of aspirates that are variably contaminated by peripheral blood. How such defined cell cycle status correlates with outcome is controversial and, as previously discussed, some of this controversy may arise from that fact that cell cycle analysis has not been performed using biopsies where G_0, in-cycle and $S/G_2/M$ populations could be defined. Furthermore, there is limited information on how cell cycle status in AML relates to well-defined biological, cytogenetic and molecular risk-factors.

In chapter 4, a cohort of patients with a new diagnosis of AML and with available biopsies taken prior to treatment was therefore studied and the clinical outcome for this cohort defined according to some of the major known prognostic factors including age, presenting WCC, cytogenetics, and *NPM1*/*FLT3* mutational status. This chapter describes the results of studies using the biopsies from this patient cohort to investigate the potential heterogeneity in cell cycle status between patients, and the correlation with clinical features and outcomes.

The specific aims of the studies performed in this chapter were to:

1. Define the heterogeneity in expression of DNA replication licensing proteins and the cell cycle status in cells from bone marrow biopsies from the defined UCLH cohort.
2. Assess how the expression of DNA replication licensing proteins and cell cycle status relates to well defined biological, molecular, and cytogenetic features.
3. Assess how the expression of DNA replication licensing proteins and cell cycle status relates to response to treatment, relapse, and overall clinical outcome.

5.2 Materials and methods

5.2.1 Patient cohort

All patients presenting with newly diagnosed AML to University College London Hospital between 1st January 2003 and December 31st 2011 and who had had a diagnostic bone marrow biopsy performed prior to treatment, as described in Chapter 4, were included in this study. Patient demographic, clinical, molecular data, and outcome data were described in Chapter 4.

5.2.2 Immunohistochemistry (IHC)

Serial sections of formalin-fixed paraffin-embedded BMTs were processed and stained as described in chapters 2 and 3 using antibodies to MCM2 to assess non-quiescent cells, regardless of whether or not they were actively cycling (positive in G_1, S, G_2 and M but not G_0), MIB-1 (equivalent to Ki-67) for actively proliferating cells (positive in a proportion of cells in G_1, and cells in S, G_2 and M but not G_0) and geminin for cycling cells that have progressed beyond G_1 (positive in S/G_2/M but not G_1 and G_0), as well as

cleaved caspase 3 to assess the proportion of cells undergoing apoptosis. Primary antibodies were used at the following concentrations: MCM2 (1:3000), Geminin (1:200), MIB-1 (1:90), and Cleaved Caspase 3 (1:300). The MIB-1/MCM2 ratio was calculated as a measure of the number of cells arrested in G_1 and the geminin/MIB-1 ratio as a surrogate for the speed at which cells were cycling. Tonsil tissue containing both glandular and epithelial components was processed at the same time and used as a positive control.

5.2.3 Validation of an automated counting system

This was an iterative process and was performed with supervision and assistance from Dr Marco Loddo (Department of Pathology, UCL, London, UK and Oncologica®, Cambridge, UK). The proportion of positive blast cells (the labelling index, LI) for each of the primary antibodies was first determined manually on 30 patient biopsies. Digital files of all biopsy sections were created using a Leica SCN400 scanner (Leica, Wetzlar, Germany). At least three separate representative areas from different sections of the biopsy were selected and JPEG files created and printed. The LI was then determined after counting a minimum of 1000 cells, with stromal cells, megakaryocytes, lymphocytes and late erythroid cells excluded by morphological criteria. The digital images obtained for each primary antibody from these 30 biopsies were then evaluated using the Ariol® automated scanning microscope and image analysis system (Turashvili *et al,* 2009). The system was trained to differentiate and enumerate positive and negative cells and to exclude stromal cells, megakaryocytes, lymphocytes and late erythroid cells using criteria for colour, size and shape. The gates for positive and negative cells according to colour, size and shape were adjusted until digital analysis of images matched the results achieved by manual counting in areas of high and low staining intensity, including paratrabecular areas, and there were no obviously negative cells counted as positive and no obviously positive cells that were not detected as such.

Having trained the system, new areas of the same 30 biopsies were then assessed. These were assessed manually and using the automated system, counting at least 1000 cells per IHC marker per biopsy, and positive cells expressed as a proportion of total blast cells (the LI). These paired analyses were tested for agreement by correlation and by Bland-Altman analysis. In addition, for these same 30 biopsies, 5 different areas from different parts of the biopsy including paratrabecular areas were selected, assessed for LI, and the consistency between readings examined by standard error of the mean (SEM), standard deviation (SD), and the coefficient of variation (CV). The total LI calculated from all positive and negative cells across the five areas from these biopsies was taken forward for the final cohort analysis. Automated analysis was also performed on the biopsy samples from the remaining 151 patients. Again, where multiple areas were assessed for a biopsy, the LI reflects the total positive and negative cells from all areas examined.

5.2.4 Haematopoietically normal bone marrow biopsies

A series of 10 haematopoietically normal bone marrow biopsies was provided by Dr Teresa Marafioti (Consultant Haematopathologist, Department of Pathology, UCLH, London, UK). These had been assessed as histologically normal during the routine work up of patients with a new diagnosis of follicular NHL. They were stained and manually assessed for each of the specific markers as previously described.

5.2.5 Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software, 'R' version 3.1.1. and SPSS version 22 as before. Consistency within a biopsy sample was assessed using SD, SEM, and CV as above. Correlations were calculated using Spearman's rank correlation coefficient. A Bland-Altman analysis was performed to assess agreement between the two methods. Differences between two cohorts were calculated using the Mann-Whitney-U test, and between more than two cohorts using the Kruskal-Wallis

test. Survival data was calculated using Kaplan-Meier estimates, with definitions of CR, OS, and DFS as previously described (see section 4.2.1), and differences were assessed using the log-rank (Mantel-Cox) test. All these survival tests were performed using Graphpad Prism 6 software. 'R' was used for calculating the CIR using a competing risks analysis, with relapse and non-relapse mortality as competing events from the time of first remission, using the cmprsk package as previously described (Scrucca *et al*, 2007). Nominal and binary logistic regression multivariate analysis was performed using SPSS version 22. All tests were two-tailed and a P value of <0.05 was considered significant.

5.3 Results

5.3.1 Validation of the automated counting system

Following completion of the programme training as outlined in section 5.2.3, new areas from the same 30 biopsies were selected. On using the Ariol® automated system to analyse the newly selected areas, there was a good correlation with the LIs determined for these areas by the manual counting; r= 0.97 ($P<0.0001$) for MCM2, 0.98 ($P<0.0001$) for geminin, 0.99 ($P <0.0001$) for MIB-1, and 0.99 ($P<0.0001$) for cleaved caspase 3 (Figure 5.1). As correlation measures the strength of the relationship between two variables, not the agreement between them, and two methods designed to measure the same variable should agree rather than simply correlate, a Bland-Altman analysis was performed. This showed that there were good agreements between the two methods and minimal apparent bias (Figure 5.2,

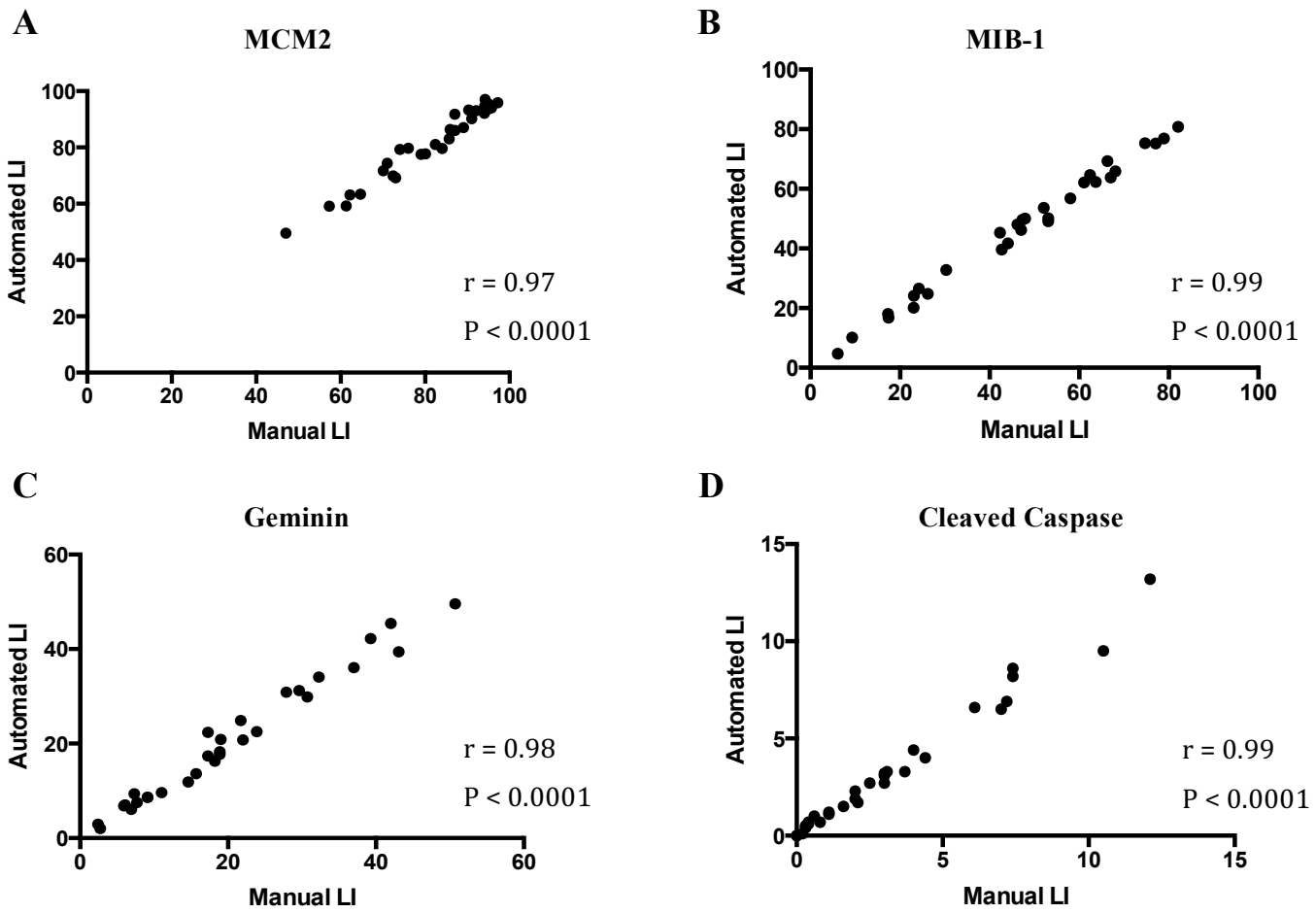


Figure 5.1 Correlation between the manual and automated counts performed on 30 biopsies. (A) MCM2, (B) MIB-1, (C) geminin, and (D) cleaved caspase. Assessed using Spearman's rank correlation co-efficient.

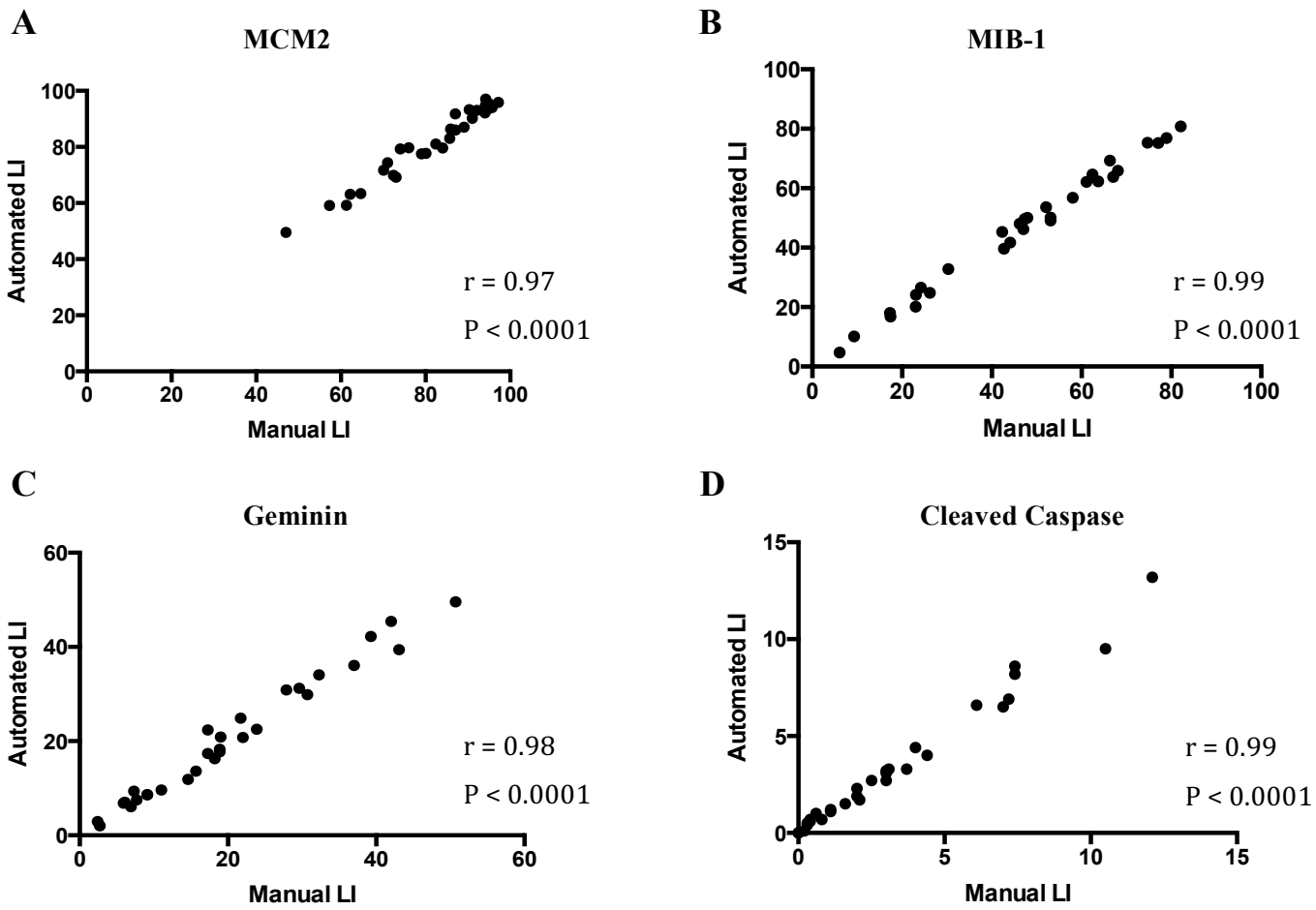


Figure 5.2 Bland-Altmann analysis of the manual and automated LI performed on 30 biopsies. (A) MCM2, (B) MIB-1, (C) geminin, and (D) cleaved caspase. Difference = (Manual LI – Automated LI). Average = (Manual LI + Automated LI)/2.

Table 5.1 Overall results of the Bland-Altman analysis to assess agreement between the manual and automated counting systems.

Protein	Bias	Standard Deviation of bias	95% limits of agreement
MCM2	-0.37	2.39	-5.05 – 4.30
MIB-1	0.27	2.14	-3.92 – 4.46
Geminin	-0.13	1.80	-3.66 – 3.40
Cleaved Caspase	-0.09	0.45	-0.98 – 0.80

A negative number shows bias towards a higher number in the automated LI, and a positive number shows bias towards a higher number in the manual LI.

Table 5.1). For example, for MCM2, the bias towards a higher result from automated assessment was 0.17 and for geminin was 0.13, with SDs of bias of 2.39 and 1.8 respectively. In contrast, for MIB-1 there was a bias towards a higher result by manual assessment of 0.27, with an SD of 2.17. It should be appreciated that these results do not suggest that either method gives a better or more accurate result, simply that they generally agree. It was therefore concluded that the automated counting method was able to provide LI results that were consistent with careful manual assessment and could therefore be used to assess the remainder of the cohort and allow rapid assessment of large numbers of cells.

5.3.2 Consistency of LI within a biopsy sample

Measurements from different areas of the same biopsy were consistent, with small values for SEM, SD, and CV (Figure 5.3, Tables 5.2-5). It can be seen that, even where the CV% was apparently high, e.g. biopsy 2 for cleaved caspase where the CV% was 223% (Table 5.5), the actual difference between the LIs was small. In this

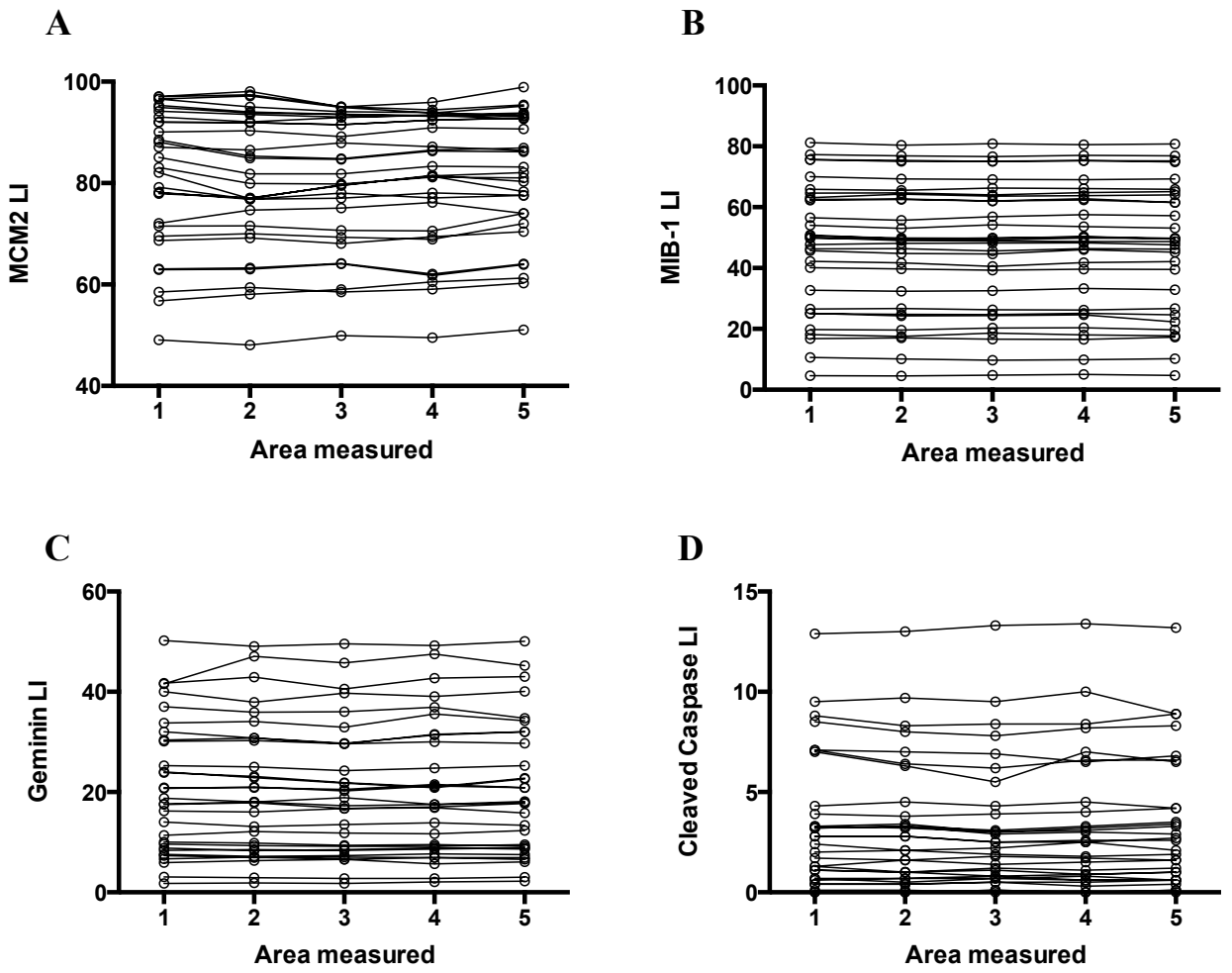


Figure 5.3 Repeated measures of LI taken from five different areas of 30 biopsies. (A) MCM2, (B) MIB-1, (C) geminin, (D) cleaved caspase.

Table 5.2 Mean LI, SEM, SD, and CV% for repeated MCM2 measurements taken from different areas of 30 biopsies.

Biopsy No.	Mean LI	SEM	SD	CV%
1	49.54	0.49	1.1	2.22
2	59.14	0.81	1.81	3.06
3	59.16	0.33	0.75	1.26
4	63.14	0.41	0.92	1.45
5	63.36	0.38	0.85	1.35
6	69.16	0.38	0.86	1.24
7	69.94	0.54	1.22	1.74
8	71.68	0.61	1.37	1.92
9	74.42	0.68	1.52	2.05
10	77.54	0.26	0.59	0.76
11	77.74	0.43	0.96	1.24
12	79.26	0.8	1.78	2.25
13	79.64	0.96	2.15	2.7
14	79.66	0.95	2.13	2.67
15	81	0.6	1.33	1.64
16	83.04	0.61	1.36	1.64
17	86.04	0.61	1.36	1.58
18	86.4	0.65	1.45	1.68
19	87.02	0.27	0.61	0.7
20	90.24	0.3	0.66	0.73
21	92.12	0.22	0.5	0.54
22	92.12	0.22	0.5	0.54
23	93.04	0.28	0.63	0.68
24	93.34	0.25	0.57	0.61
25	93.74	0.31	0.69	0.73
26	93.98	0.35	0.78	0.83
27	94.56	0.59	1.31	1.38
28	95.54	0.61	1.36	1.43
29	95.86	0.57	1.28	1.34
30	97	0.71	1.58	1.63
Median Range	84.54 (49.54 - 97)	0.55 (0.22 - 0.96)	1.16 (0.5 - 2.15)	1.44 (0.54-3.06)

Table 5.3. Mean LI, SEM, SD, and CV% for repeated MIB-1 measurements taken from different areas of 30 biopsies.

Biopsy No.	Mean LI	SEM	SD	CV%
1	4.72	0.09	0.19	4.08
2	10.1	0.15	0.34	3.36
3	16.84	0.14	0.32	1.91
4	17.98	0.19	0.42	2.34
5	19.96	0.16	0.36	1.83
6	24.14	0.48	1.07	4.45
7	24.84	0.09	0.21	0.83
8	26.48	0.1	0.23	0.86
9	32.76	0.16	0.36	1.09
10	39.64	0.13	0.3	0.75
11	41.66	0.3	0.68	1.63
12	45.26	0.26	0.59	1.3
13	46.18	0.15	0.33	0.72
14	47.98	0.14	0.31	0.65
15	49.06	0.24	0.54	1.1
16	49.62	0.12	0.28	0.56
17	50.02	0.27	0.59	1.19
18	50.06	0.14	0.32	0.64
19	53.58	0.24	0.53	0.99
20	56.76	0.31	0.7	1.23
21	62.14	0.18	0.4	0.65
22	62.3	0.24	0.54	0.87
23	63.76	0.2	0.45	0.71
24	64.64	0.18	0.4	0.62
25	65.94	0.13	0.3	0.45
26	69.34	0.17	0.39	0.56
27	75.18	0.12	0.28	0.37
28	75.3	0.1	0.22	0.3
29	76.94	0.11	0.25	0.33
30	80.76	0.14	0.32	0.4
Median Range	49.82 (4.72 - 80.76)	0.16 (0.09 - 0.48)	0.36 (0.19 - 1.07)	0.87 (0.3 - 4.45)

Table 5.4. Mean LI, SEM, SD, and CV% for repeated geminin measurements taken from different areas of 30 biopsies.

Biopsy No.	Mean LI	SEM	SD	CV%
1	1.96	0.08	0.18	9.27
2	2.92	0.06	0.13	4.47
3	6.12	0.16	0.35	5.71
4	6.76	0.08	0.18	2.69
5	7	0.08	0.17	2.47
6	7.46	0.09	0.21	2.78
7	8.56	0.09	0.21	2.42
8	8.62	0.11	0.24	2.77
9	9.36	0.08	0.18	1.94
10	9.58	0.14	0.32	3.33
11	11.86	0.16	0.35	2.96
12	13.58	0.17	0.37	2.73
13	16.32	0.21	0.47	2.85
14	17.36	0.22	0.5	2.87
15	17.66	0.13	0.29	1.63
16	18.26	0.26	0.59	3.21
17	20.8	0.17	0.38	1.83
18	20.94	0.16	0.36	1.71
19	22.44	0.52	1.17	5.2
20	22.54	0.5	1.12	4.97
21	24.94	0.19	0.42	1.67
22	29.94	0.13	0.29	0.96
23	30.86	0.4	0.89	2.88
24	31.18	0.45	1.01	3.24
25	34.12	0.44	0.97	2.85
26	36.1	0.42	0.93	2.58
27	39.36	0.4	0.9	2.3
28	42.18	0.46	1.02	2.43
29	45.44	1.05	2.34	5.15
30	49.64	0.22	0.5	1.01
Median Range	17.96 (1.96 - 49.64)	0.18 (0.06 - 1.05)	0.4 (0.13 - 2.34)	2.82 (0.96 - 9.27)

Table 5.5 Mean LI, SEM, SD, and CV% for repeated cleaved caspase measurements taken from different areas of 30 biopsies.

Biopsy No.	Mean LI	SEM	SD	CV%
1	0.04	0.02	0.05	136.93
2	0.02	0.02	0.04	223.61
3	0.06	0.02	0.05	91.29
4	0.4	0.03	0.07	17.68
5	0.48	0.04	0.08	17.43
6	0.62	0.04	0.08	13.49
7	0.7	0.03	0.07	10.1
8	0.66	0.04	0.09	13.55
9	0.96	0.05	0.11	11.88
10	1.02	0.04	0.08	8.2
11	1.16	0.05	0.11	9.83
12	1.48	0.06	0.13	8.81
13	1.68	0.04	0.08	4.98
14	1.94	0.05	0.11	5.88
15	2.26	0.08	0.18	8.04
16	2.66	0.07	0.15	5.7
17	2.66	0.06	0.13	5.04
18	3.06	0.08	0.18	5.94
19	3.18	0.06	0.13	4.1
20	3.26	0.07	0.17	5.13
21	3.28	0.07	0.15	4.52
22	3.96	0.07	0.15	3.83
23	4.36	0.06	0.13	3.08
24	6.46	0.28	0.62	9.58
25	6.58	0.15	0.33	5.09
26	6.86	0.1	0.23	3.36
27	8.16	0.12	0.27	3.31
28	8.56	0.12	0.27	3.16
29	9.52	0.18	0.4	4.23
30	13.16	0.09	0.21	1.58
Median Range	2.46 (0.04-13.16)	0.06 (0.02- 0.28)	0.13 (0.04 - 0.62)	6.99 (1.58 - 223.61)

case, the repeated LI results of cleaved caspase performed on different areas were 0, 0, 0.1, 0, and 0. These results suggest that the LI was consistent and representative of the rest of the biopsy, regardless of which area was assessed.

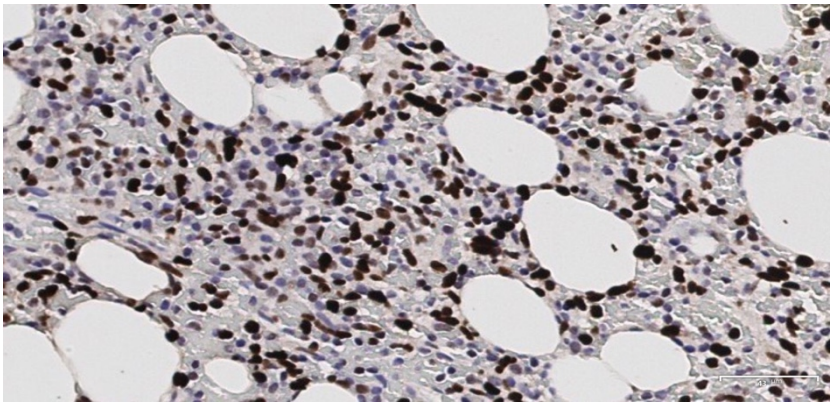
5.3.3 Cell cycle analysis and cleaved caspase staining

Analysis of DNA replication licensing proteins in all 181 patients indicated that the majority of leukaemic blasts in the BMTs were not quiescent (median LI for MCM2, 81%), and approximately one-third of the non-quiescent cells were MIB-1 positive (median LI, 32%) and therefore actively cycling (Table 5.6). The median LI for geminin was 17%, and approximately half of the actively cycling cells had progressed beyond G_1 (median geminin/MIB-1 ratio, 0.51). There was, however, considerable heterogeneity within the cell cycle profile, with wide interquartile and overall ranges. Morphological examples of cases with high and low LI for MCM2 and for geminin are shown in Figures 5.4 and 5.5 respectively. Very few blasts were undergoing apoptosis (median LI for cleaved caspase, 3%). To explore these results further, they were correlated with well-defined AML risk factors and subgroups.

Table 5.6 The median and interquartile ranges for the cell cycle markers and derived ratios for all 181 AML patients

Antigen	Median LI	Interquartile range	Overall range
MCM2	81%	69-92%	40.5 - 100%
MIB-1	32%	23-49%	0.1 - 81.7%
Geminin	17%	10-24%	0 -66.1%
Geminin/MIB-1 ratio	0.51	0.35-0.79	0 - 0.97
MIB1/MCM2 ratio	0.42	0.29 – 0.60	0 - 0.99
Cleaved caspase	2.7%	1.1-5.2%	0 - 17.2%

A



B

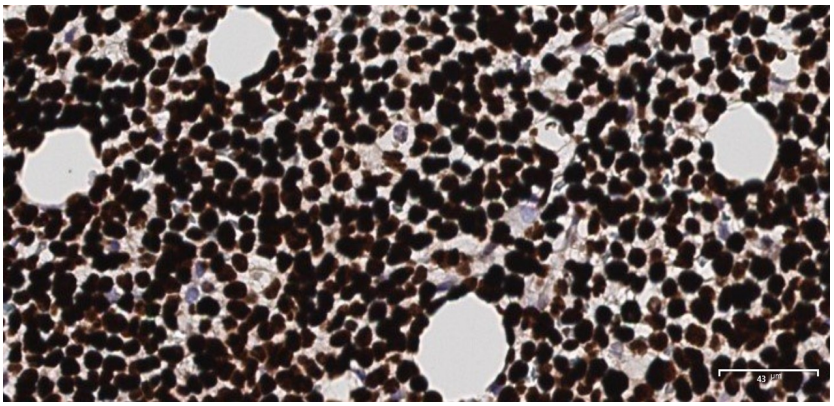
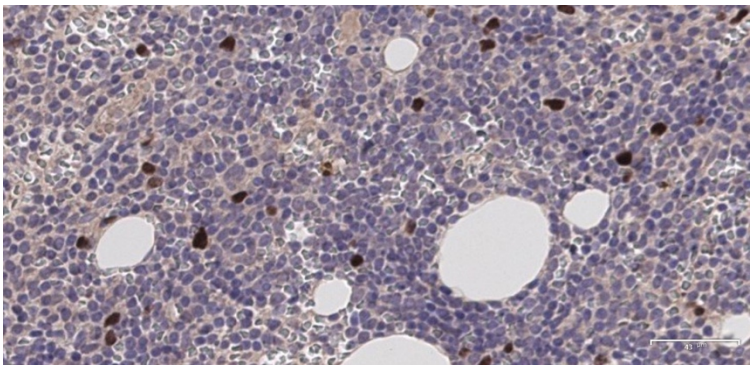


Figure 5.4 Morphological examples of cases with (A) low and (B) high LI for MCM2 (x40 magnification).

A



B

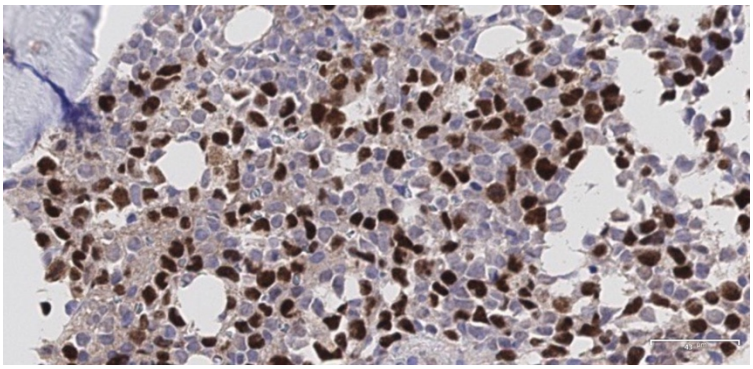


Figure 5.5 Morphological examples of cases with (A) low and (B) high LI for geminin (x 40 magnification).

5.3.4. Relationship of cell cycle profiles to biological risk factors

5.3.4.1 Relationship to age

There was no correlation between any of the cell cycle parameters and patient age at presentation (Figure 5.6A-C). In contrast, there was some evidence that the LI for cleaved caspase was positively correlated with increasing age (r=0.28, P=0.0001) (Figure 5.6D). This relationship was maintained when the results for patients with APL or sAML/tAML were excluded (r=0.3, P=0.0003 for both analyses).

5.3.4.2 Relationship to presenting white cell count

Figure 5.7 shows the relationship between the presenting WCC and the LIs for the cell cycle markers, the derived ratios MIB-1/MCM2 and geminin/MIB-1, and cleaved caspase. The presenting WCC was significantly correlated with the proportion of non-quiescent cells as defined by MCM2 positivity (r=0.4, P<0.0001) (Figure 5.7A), but there was no statistical relationship to the proportion of actively cycling cells (for MIB-1 LI, r=0.1, P=0.06) (Figure 5.7B). In contrast, it was positively correlated with the proportion of cycling cells beyond G_1 and the speed of cell cycle in cycling cells (for geminin LI, r=0.4, P<0.0001; geminin/MIB-1 ratio, r=0.3, P<0.0001) (Figure 5.7C,E). It was also inversely correlated with the proportion of apoptotic cells (for cleaved caspase LI, total cohort, r=-0.2, P=0.002; excluding APL patients, r=0.3, P<0.0001) (Figure 5.7F). These results suggest that a higher WCC may occur in the context of faster cell cycle progression rates or lower levels of apoptosis. No significant differences in cell cycle parameters or the proportion of apoptotic cells were observed between the *de novo* and secondary AML patients (Table 5.7).

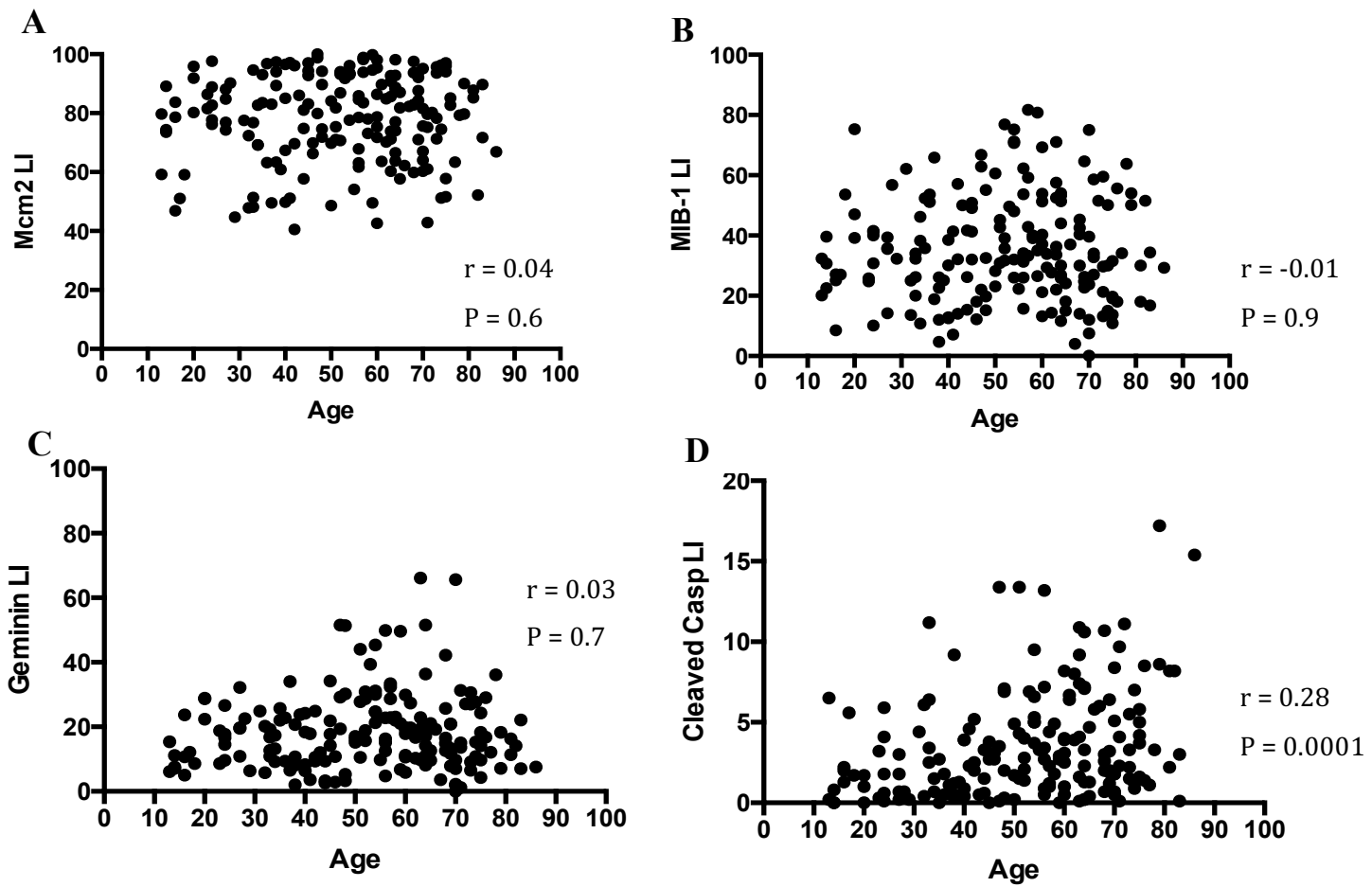


Figure 5.6 Correlation between age and the LI for the 181 patients with AML. (A) MCM2, (B) MIB-1, (C) geminin, and (D) cleaved caspase.

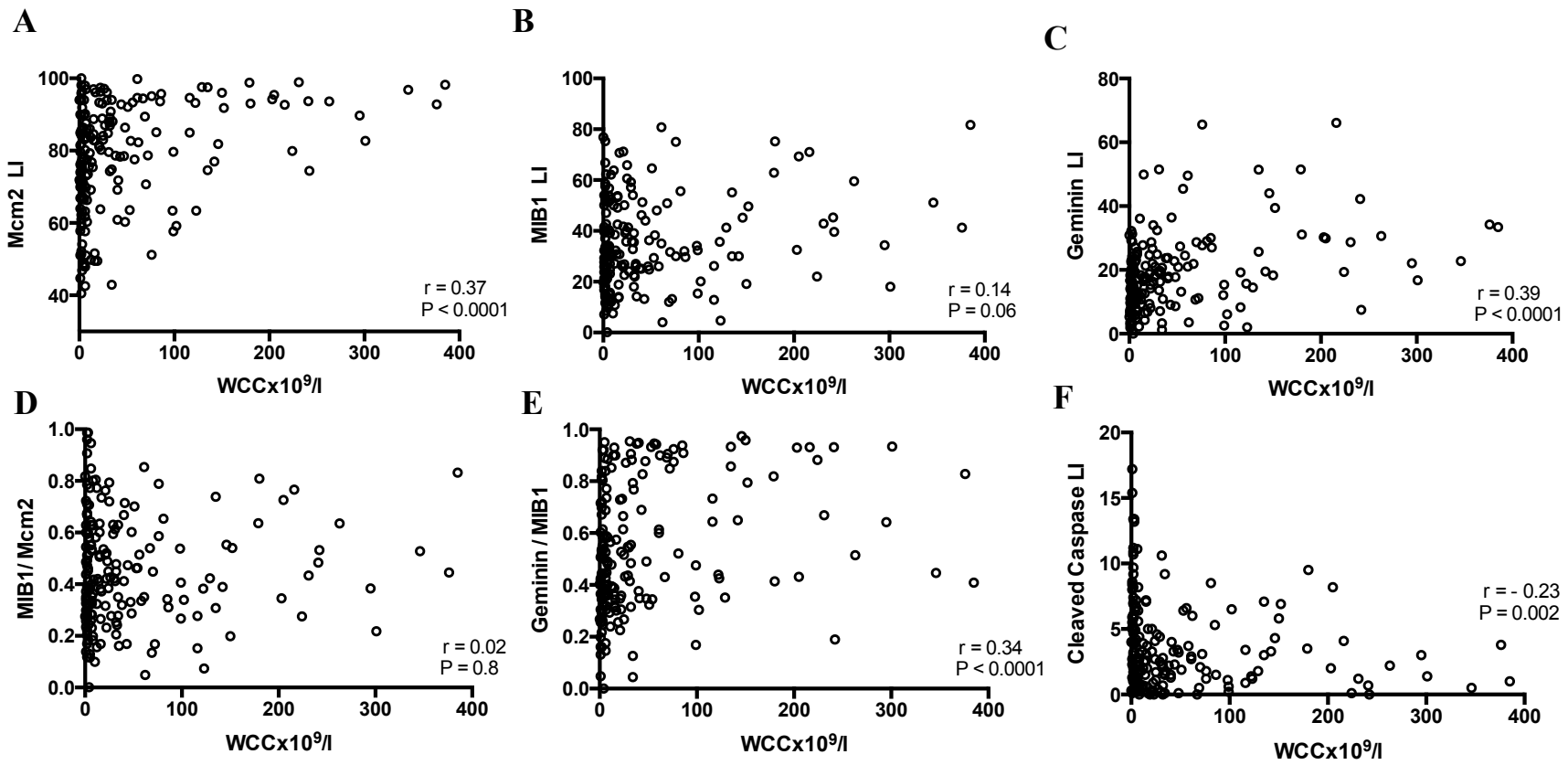


Figure 5.7 Correlation between presenting white cell count and the LI or derived ratios in the 181 patients with AML. (A) MCM2, (B) MIB-1 and (C) geminin, or the derived ratios (D) MIB-1/MCM2 and (E) geminin/MIB-1,or (F) cleaved caspase.

5.3.4.3 Relationship to FAB type

The LIs for all markers and the derived ratios were significantly different for patients with APL (FAB type M3) when compared to non-APL patients (Figure 5.8). Patients with APL had significantly fewer non-quiescent cells (median MCM2 LI, 68% vs 82%; P=0.03) (Figure 5.8A), actively cycling cells (median MIB-1 LI, 16% vs 34%; P=0.0005) (Figure 5.8B) and cells in $S/G_2/M$ (median geminin LI, 6% vs 18%, P<0.0001) (Figure 5.8C), and a greater proportion of cells in a G_1-arrested state (MIB-1/MCM2 ratio, 0.27 vs 0.45; P=0.002) (Figure 5.8D). In addition, they had a significantly lower median geminin/MIB-1 ratio (0.33 vs 0.52, *P*=0.005) (Figure 5.8E), a result that implies a longer average duration of G_1 in actively cycling cells. The APL patients also had a significantly lower proportion of cleaved caspase-labelled cells (median, 0.9 vs 2.9, P=0.003) (Figure 5.8F).

With M3 excluded, when other FAB types were assessed for differences between the groups with a sufficient number of patients (M0, M1, M2, M4, and M5), there were no statistically significant differences between the FAB types for any of the cell cycle markers, derived ratios, or cleaved caspase (Table 5.8).

5.3.4.4 Relationship to Cytogenetic Risk Groups

There were no significant differences in cell cycle parameters between the cytogenetic risk groups in the non-APL patients, either for the proportion of non-quiescent cells (median MCM2 LI for favourable-, intermediate- and adverse-risk patients, 80% vs 85% vs 78% respectively; P=0.06), actively cycling cells (median MIB-1 LI, 32% vs 34% vs 27%; P=0.1), or cells expressing geminin (median LI, 18% vs 19% vs 17%; P=0.07). Similarly, neither the proportion of non-quiescent cells that were actively cycling nor the cell cycle duration differed significantly (MIB-1/MCM2 ratio, 0.42 vs 0.46 vs 0.37; P=0.3; geminin/MIB-1 ratio, 0.54 vs 0.5 vs

Table 5.7 LI according to subtype of leukaemia: *de novo*, sAML, or tAML.

AML type	MCM2 LI	MIB-1 LI	Geminin LI	MIB-1/MCM2	Geminin/MIB-1	Cleaved Caspase LI
De novo	81.7 (42.6-99.8)	32.7 (0.1-81.7)	16.8 (0-66.1)	0.44 (0.00-0.99)	0.49 (0.00-0.97)	2.7 (0-15.4)
sAML	79.3 (51.6-100)	26 (10.9-59.2)	18.9 (4.3-65.6)	0.34 (0.13-0.80)	0.64 (0.13-0.94)	3.0 (0.1-7.2)
tAML	78.3 (40.5-94)	34.3 (12-66.8)	15.3 (5.3-32.4)	0.47 (0.11-0.91)	0.52 (0.16-0.94)	2.9 (0.1-7.4)
P	0.74	0.77	0.86	0.57	0.33	0.97

Values given are medians and ranges.

Abbreviations: sAML; secondary AML, tAML; therapy related AML

Table 5.8 LI according to FAB subtype.

FAB type (no.)	MCM2	MIB-1	Geminin	MIB-1/MCM2	Geminin/MIB-1	Cleaved Caspase
M0 (12)	76.8 (42.9-95.9)	25.9 (4.7-75.3)	13.3 (1.2-27)	0.35 (0.07-0.96)	0.4 (0.04-0.91)	1.4 (0-9.7)
M1 (39)	85.7 (60.3-99.8)	30.7 (4.0 - 71.0)	18.3 (3.3-66.1)	0.35 (0.05-0.77)	0.59 (0.13-0.96)	2.6 (0-9.2)
M2 (65)	77.5 (42.6-97.0)	33.9 (20-80.8)	17.3 (1.9-49.9)	0.46 (0.29-0.98)	0.4 (0.05-0.95)	3.7 (0-17.2)
M3 (15)	67.9 (44.7-98.1)	15.7 (0.1-50)	5.8 (0-22)	0.27 (0.1-72.2)	0.36 (0-0.70)	0.9 (0.2-3.9)
M4 (17)	83 (60.9-98.9)	35.8 (25.1-75.2)	22.5 (7.5-65.6)	0.46 (0.35-0.8)	0.53 (0.19-0.95)	1.5 (0-9.5)
M5 (25)	84 (57.7-98.2)	37.3 (12-81.7)	22.9 (9.1-51.5)	0.47 (0.13-0.83)	0.55 (0.27-0.95)	2.2 (0-10.6)
P (all)	<0.0001	0.002	<0.0001	0.002	0.006	0.002
P (no M3)	0.07	0.12	0.07	0.1	0.08	0.1

P values given represent a Kruskal-Wallis test across the groups with and without M3 patients. Values given are medians and ranges.

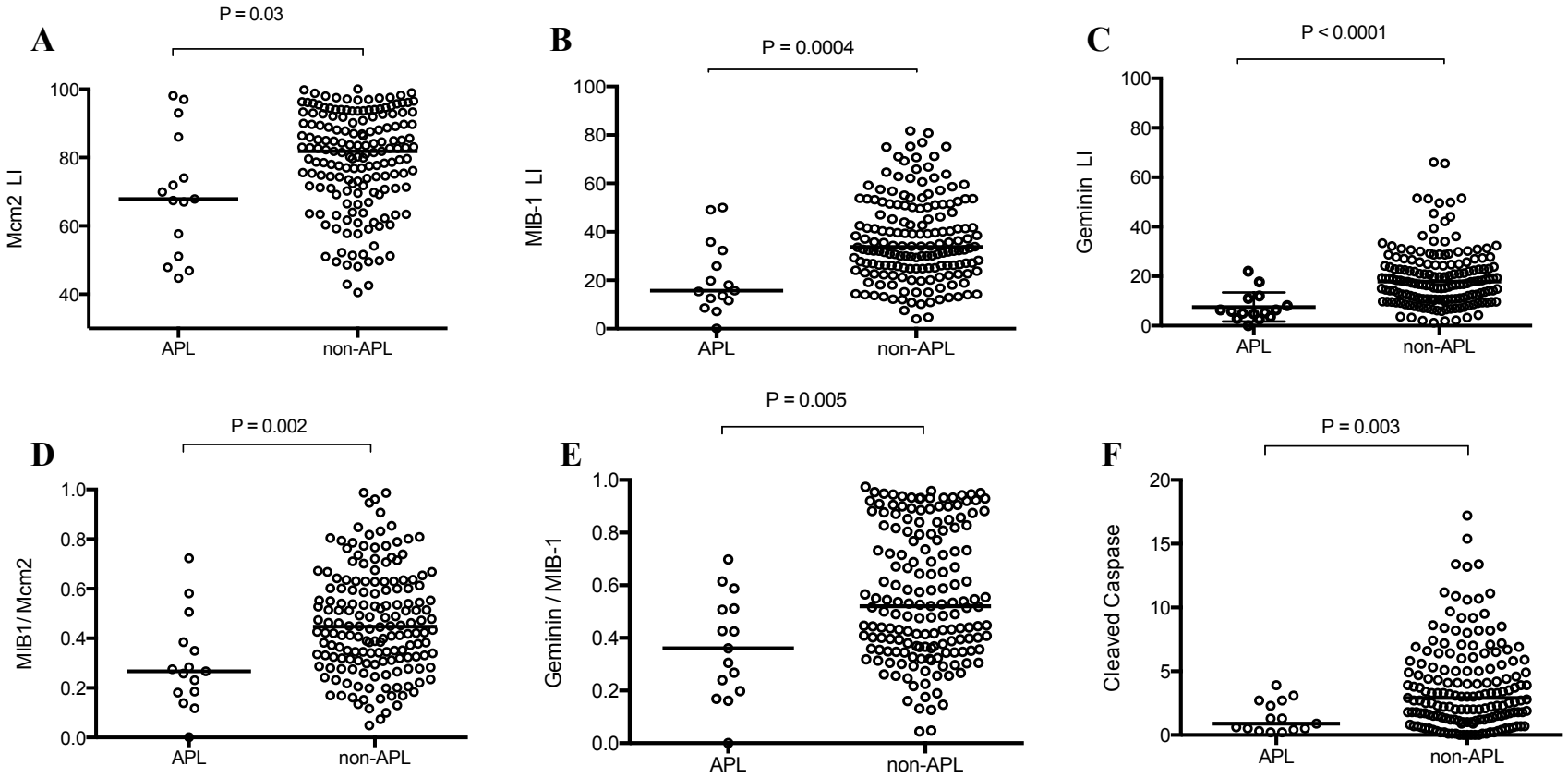


Figure 5.8 Difference in the LI and derived ratios between APL and non-APL patients. (A) MCM2, (B) MIB-1, (C) geminin, (D) MIB-1/MCM2, (E) geminin/MIB-1, (F) cleaved caspase.

0.53; P=0.4). However, the proportion of apoptotic cells was slightly, but significantly, higher in the poorer cytogenetic risk patients (median cleaved caspase LI, 1.8% vs 2.7% vs 3.3%, respectively; P=0.03).

5.3.4.5 Relationship to molecular genotype in patients with intermediate-risk cytogenetics

The association of *NPM1* and *FLT3*^{ITD} mutation status was then assessed in the 85 patients with intermediate-risk cytogenetics whose molecular genotype was known. As discussed in chapter 4, these mutations were predominantly associated with this risk group, and it was here that their prognostic implications were most relevant.

The proportion of non-quiescent cells was marginally higher in the *NPM1*^{MUT} cases compared to those with *NPM1*^{WT} (median MCM2 LI, 89% vs 85%; P=0.03). Although the proportion of actively cycling cells was also higher, the difference was not significant (median MIB-1 LI, 40% vs 34%; P=0.4). However, the proportion of cycling cells that had progressed beyond G_1 was significantly higher in the *NPM1*^{MUT} cases (median geminin LI, 24% vs 15%; P=0.002) (Figure 5.9A), as was the geminin/MIB-1 ratio (0.68 vs 0.40; P=0.003) (Figure 5.9B). These results indicate that although *NPM1*^{MUT} and *NPM1*^{WT} patients have a similar proportion of actively cycling cells, a feature of *NPM1*^{MUT} cases is that cycling cells spend a smaller proportion of time in G_1, a surrogate for the speed of cycling.

Non-quiescent cells were slightly higher in *FLT3*^{ITD} than *FLT3*^{WT} patients (median MCM2 LI, 92% vs 84%; P=0.03) but the proportion of actively cycling cells and of cells in S/G_2/M was similar (median MIB-1 LI, 36% vs 36%; *P*=0.77; median geminin LI, 21% vs 19%; P=0.5). The geminin/MIB-1 ratio was also not significantly different (0.48 vs 0.52; P=0.8). The proportion of cells positive for cleaved caspase was not influenced by either the *NPM1* or *FLT3* genotype (P=0.2 and P=0.4 respectively).

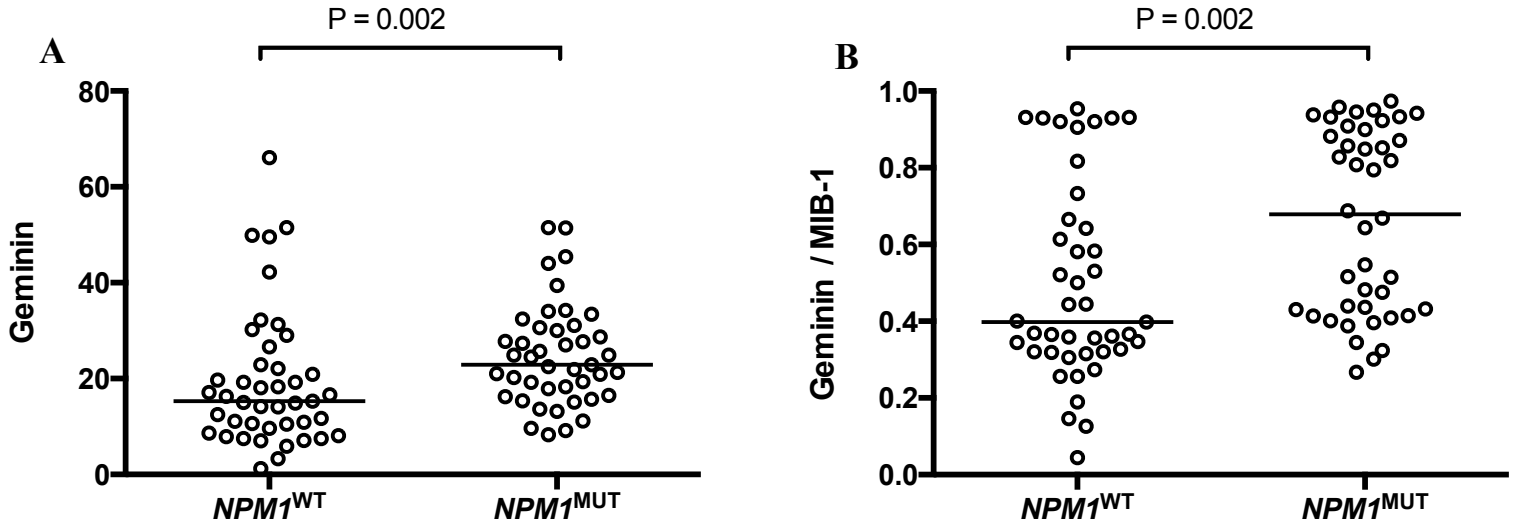


Figure 5.9 Comparison between intermediate-risk patients *NPM1*^{WT} and *NPM1*^{MUT} patients according to (A) geminin expression and (B) geminin/MIB-1 ratio.

5.3.5 Comparison between biopsies from AML patients and haematologically normal biopsies

The results of cell cycle analysis performed using biopsies from 10 individuals with demonstrably normal bone marrows are shown in Table 5.9, together with the results from the 166 AML patients with non-APL disease. There was a trend towards a greater proportion of cells being licensed for replication (MCM2-positive) in the biopsies from the AML patients than the haematologically normal biopsies (81.8 vs 75.5, P=0.07), although there was no difference in the proportion of geminin-positive cells (17.9 vs 17.1, P=0.87), implying that an equivalent proportion of cells had progressed beyond G_1 in the histologically normal bone marrows. However, a striking feature of the biopsies from patients with AML as compared to the haematologically normal biopsies was a lower proportion of cells actively cycling, as evidenced by a lower MIB-1/MCM2 ratio (33.8 vs 63.7, P<0.0001), although a significantly higher geminin/MIB-1 ratio (0.52 vs 0.27, P<0.0001) suggested that those cells that were actively cycling were doing so at an increased rate. Of note, the range of values was greater in the marrow from individuals with AML.

Table 5.9 The median and overall range for the cell cycle markers and derived ratios for the 166 patients with non-APL disease as compared to haematologically normal biopsies.

	Median (range)		P
	AML (non-APL) (n=166)	**Normal biopsy (n=10)**	
MCM2 LI	81.8 (40.5 - 100)	75.5 (64.4 - 80.3)	0.07
MIB-1 LI	33.8 (4 - 81.7)	63.7 (51.4 - 72)	< 0.0001
Geminin LI	17.9 (1.2 - 66.1)	17.1 (11.2 - 24.4)	0.87
MIB-1/MCM2	0.45 (0.05 - 98.7)	0.86 (0.73 - 0.98)	< 0.0001
Geminin/MIB-1	0.52 (0.045 - 0.99)	0.27 (0.18 - 0.35)	< 0.0001

5.3.6 Relationship between cell cycle status and response to therapy

In the 140 non-APL patients that received intensive induction therapy, 66% achieved a CR after one cycle of chemotherapy, and neither the proportion of non-quiescent cells nor of actively cycling cells differed between those who did or did not achieve CR (MCM2 LI, 83% vs 75% respectively, P=0.2; MIB-1 LI, 34% vs 37%; P=0.9). Nevertheless, patients who achieved an early remission did have a significantly higher proportion of cells beyond G_1 (geminin LI, 19% vs 13%; P=0.004) and a higher geminin/MIB-1 ratio (0.54 vs 0.41; P=0.0002). This relationship was still statistically significant when CR at any time was considered, which included 71% of the cohort (geminin LI, 19% vs 12%; P=0.02; geminin/MIB-1 ratio, 0.52 vs 0.42; P=0.02). However, further inspection of the response data revealed that the CR rates were confounded by an excess of IDs in those patients with higher geminin/MIB-1 ratios (19% vs 6% for above and below the median respectively, P=0.02). In multivariate analysis of the total cohort considering biological, cytogenetic and molecular characteristics, the factors predicting for ID were WCC (P=0.01) and age (P=0.02), with the impact of the geminin/MIB-1 ratio not achieving significance (P=0.1). However, if IDs were excluded, the factors significantly predicting for attainment of CR after one cycle of therapy were the presence of an *NPM1* mutation (P=0.001), and the geminin/MIB-1 ratio (P=0.01). Geminin could also predict for achievement of CR (P=0.01), but only if the geminin/MIB-1 ratio was removed from the model. Factors predicting for RD in this multivariate model were increasing age (P=0.01), and adverse cytogenetics P=0.01).

When the patients were grouped according to quartiles of the geminin/MIB-1 ratio, it was apparent that there was an association between this ratio and the achievement of CR after one cycle of therapy, with the highest CR rate (74%) in the quartile with the highest geminin/MIB-1 ratio (Q1; median, 0.81) and the lowest CR rate (54%) in those with the lowest ratio (Q4; median, 0.30), although this difference did not reach significance (P=0.08 across all quartiles) (Table 5.10). IDs were, however, significantly higher in Q1 (26%) and lower in Q4 (6%; P=0.01 across all groups), and this is likely to relate to the fact that patients in Q1 had a much higher presenting WCC, which is a known risk factor for ID and an independent adverse risk factor for ID in this study. In fact, of the nine IDs in Q1 patients, seven had a WCC above the median value of 56

$x10^9$/l for this quartile. Furthermore, six of these seven patients were over the age of 60 years, and four of the nine patients with early deaths had secondary leukaemia. When the induction mortality as well as the CR rate was taken into account, there was a highly significant relationship between RD and the geminin/MIB-1 ratio. In Q1, every patient who was alive after the first course of induction therapy was in CR compared to only 58% in Q4 (P=0.0002) (Table 5.10). Considering best response at any time, the values for RD were 0% in Q1 and 24% in Q4 patients (P=0.02). It is notable that the incidence of *NPM1* mutations was much higher in Q1 than in Q4, and it is not possible to clearly separate the impact on chemo-resistance of the cell cycle profile per se and the *NPM1* mutational status.

5.3.7 Relationship between cell cycle status, cleaved caspase staining and overall survival

The OS in the 140 intensively treated patients was then assessed according to cell cycle markers. For each marker or derived ratio, two groups were defined by virtue of being above or below the median, and the outcome compared. There was no difference in OS or DFS for any of the analyses (Figure 5.10).

5.3.8 Relationship between geminin/MIB-1 ratio and long-term outcome

The CIR and OS at 3 years in the patients who received intensive induction therapy was 47% and 41% respectively. The CIR was significantly different between the quartiles of the geminin/MIB-1 ratio; it was highest (74%) in Q4 patients with the lowest geminin/MIB-1 ratio (P=0.01) (Figure 5.11). Although the difference in OS

Table 5.10 Patient characteristics and outcome according to quartiles of the geminin/MIB-1 ratio in non-APL patients receiving intensive induction therapy.

	Total cohort (n=140)	Q1 (n=35)	Q2 (n=35)	Q3 (n=35)	Q4 (n=35)	P
Geminin/MIB-1						
Mean	0.56	0.90	0.64	0.43	0.27	
Median	0.52	0.81	0.61	0.43	0.30	
Range	0.04-0.97	0.82-0.97	0.52-0.81	0.37-0.51	0.04-0.36	
Cell cycle parameters						
Geminin, median LI	18%	26%	20%	18%	9%	0.0001
MIB-1, median LI	34%	29%	34%	41%	40%	0.01
MCM2, LI	83%	83%	82%	84%	75%	0.3
MIB-1/MCM2 ratio	0.45	0.35%	0.42	0.54	0.52	0.001
Cleaved caspase, median LI	2.7%	2.3%	3.0%	2.7%	2.3%	1.0
Median age, yrs	54	54	57	47	50	0.2
Median WCC, $x10^9/l$	22	56	8	12	15	0.0004
Cytogenetics, n (%)						
Favourable	13	3 (23%)	4 (31%)	4 (31%)	2 (15%)	
Intermediate	80	26 (33%)	16 (20%)	20 (25%)	18 (23%)	
Adverse	23	2 (9%)	7 (30%)	7 (30%)	7 (30%)	
Unknown	24	4 (17%)	8 (33%)	4 (17%)	8 (33%)	NS
Genotype						
NPM1^{MUT}	43/128 (34%)	17/30 (57%)	8/30 (27%)	15/33 (45%)	3/35 (9%)	0.001
FLT3^{ITD}	29/129 (22%)	9/30 (30%)	7/31 (23%)	8/33 (24%)	5/35 (14%)	0.2
Response to therapy						
Induction deaths	12%	26%	11%	6%	6%	0.01
CR post 1 cycle	66%	74%	69%	66%	54%	0.1
CR at any time	72%	74%	71%	71%	71%	1.0
Alive but not in CR						
Post 1 cycle	23%	0%	23%	31%	42%	0.0002
At any time	16%	0%	19%	23%	23%	0.02
Long-term outcome						
CIR at 3 yrs	49%	48%	44%	29%	73%	0.01
Median time to relapse, (days)	335	304	452	319	265.5	0.4
OS at 3 years	41%	32%	46%	52%	34%	

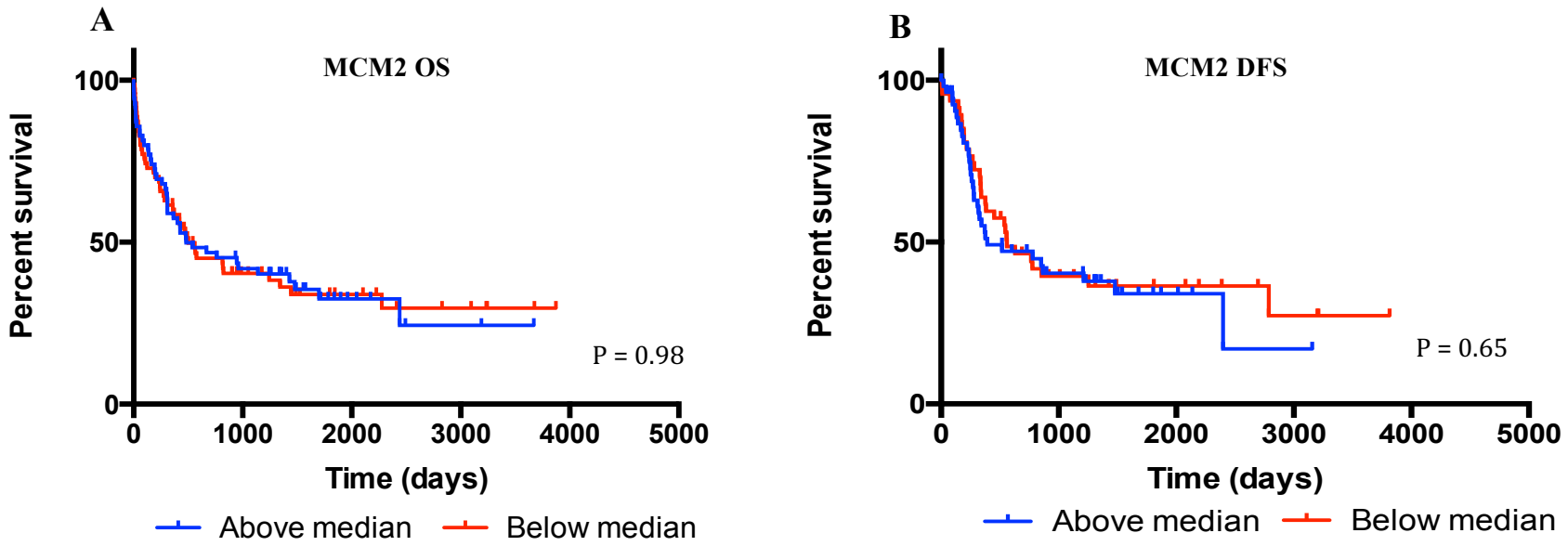


Figure 5.10 Overall survival (OS) and disease free survival (DFS) for the 140 intensively treated non-APL patients stratified according to the median values. (A, B) MCM2, (C, D) MIB-1, (E, F) geminin, (G, H) MIB-1/MCM2 ratio, and (I, J) geminin/MIB-1 ratio.

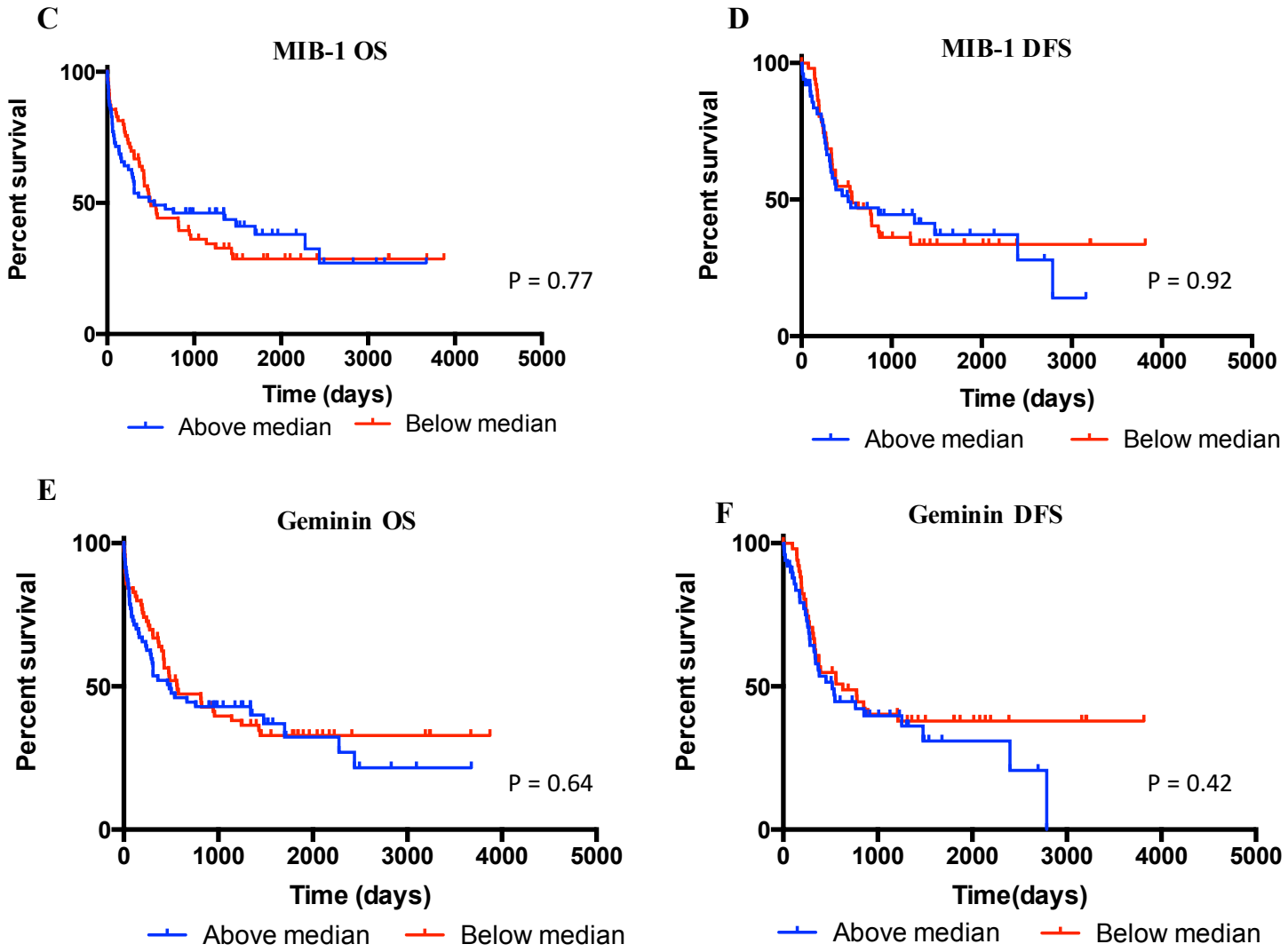


Figure 5.10 (continued)

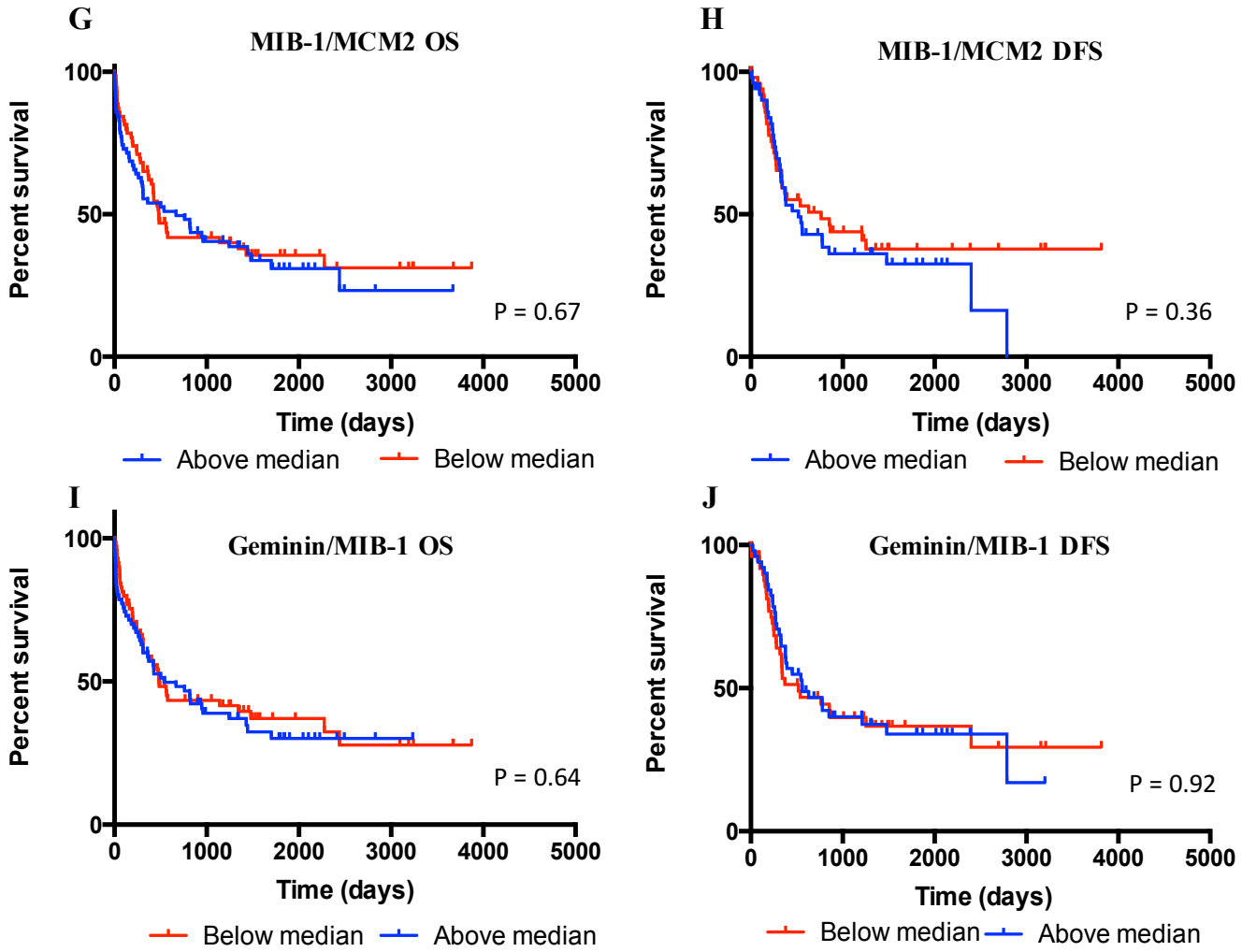


Figure 5.10 (continued)

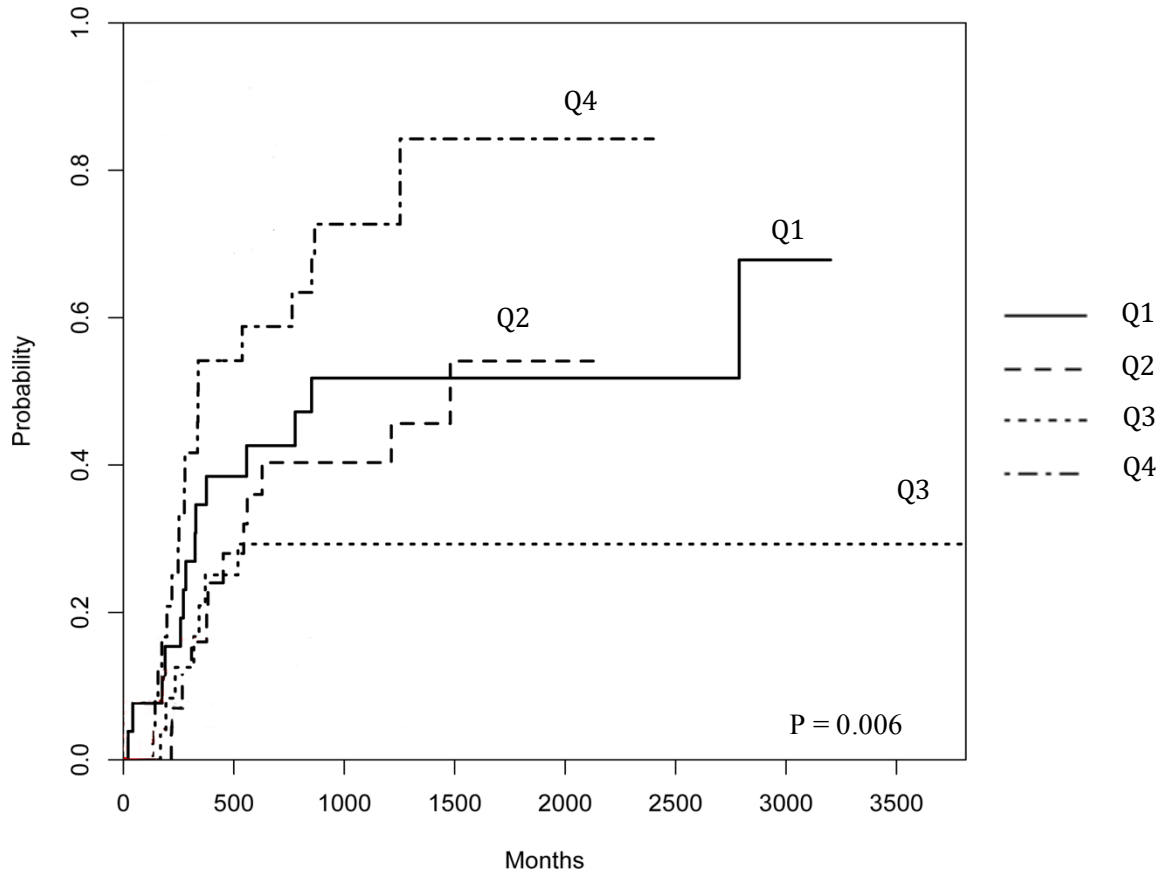


Figure 5.11 Cumulative incidence of relapse stratified according to quartiles of the geminin/MIB-1 ratio. Q1, highest; Q4, lowest.

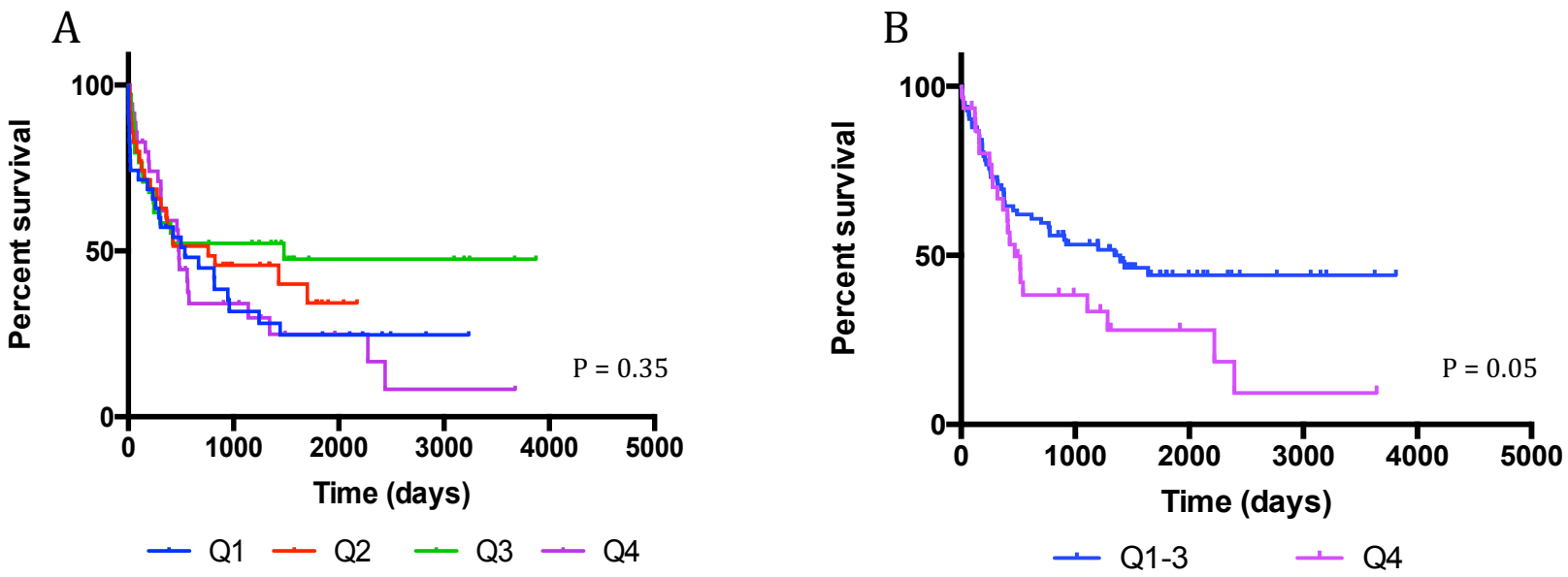


Figure 5.12 Overall survival stratified according to the geminin/MIB-1 ratios. (A) OS for Q1 (highest) to Q4 (lowest), (B) OS from the start of the second cycle of intensive chemotherapy using the geminin/MIB-1 ratio at diagnosis and comparing the highest 3 quartiles (Q1-3) with the lowest quartile (Q4).

between the quartiles was not significant (Figure 5.12A), this was again confounded by the higher rate of ID in the Q1 patients. In view of the high relapse rate in the Q4 patients, an exploratory landmark analysis was performed from the time at which patients received their second cycle of intensive chemotherapy, comparing Q4 (n=31) with Q1-3 patients (n=83). The difference in survival between these groups was borderline significant (P=0.05) (Hazard ratio by log rank analysis, 1.65; 95% confidence intervals, 0.996 – 3.126). Median survival was 472 vs 1346 days in Q4 and Q1-3 respectively (Figure 5.12B).

5.4 Discussion

This chapter presents results on the association between the expression of DNA replication licensing proteins and the cell cycle status and outcome in a cohort of 181 AML patients. The patients were from a single centre but, as shown in chapter 4, the characteristics of the cohort with regard to age, cytogenetics, mutational status and response to treatment were similar to other series reported in the literature, with particular reference to the UK MRC AML trials. An important feature of the study was that the cell cycle analysis of the leukaemic cells was determined solely using IHC analysis on cells present in BMTs, which was essential in order to avoid the issue of blood contamination of BMAs that was demonstrated in chapter 3 to be critical to the results obtained.

Before analysis of the whole cohort, two key points were demonstrated. Firstly it was shown that large number of marrow cells could be assessed rapidly by using an automated system. This system, when trained, was shown to give results consistent with manual counting on multiple biopsies. Both central medullary spaces and paratrabecular areas were tested. It was also assessed whether the area of a biopsy gave a different result in any individual patient. Sampling of five different areas, again including paratrabecular areas, showed consistent results between different areas of the same patient, with generally low levels of SEM, SD, and CV%. This is important as it suggests that the percentage of cells expressing the different replication licensing

proteins is consistent throughout the sample. It also suggests that for smaller biopsies, where fewer cells are available or suitable areas of tissue can only be identified in certain areas of the biopsy, that the results will still be representative.

Having established the automated system, the remaining samples in the cohort were assessed. The results highlight many important points about the expression of DNA replication licensing proteins and the cell cycle status in patients with AML. For example, they demonstrate the extensive heterogeneity seen between patients. When this heterogeneity was correlated with clinical features, there were a number of associations that could be identified. Firstly, a more proliferative profile, with fewer quiescent cells (MCM2 negative), more cells in $S/G_2/M$ (geminin positive), and a faster cycling rate (higher geminin/MIB-1 ratio), was associated with a higher presenting WCC, as was a lower percentage of apoptotic cells (cleaved caspase positive). Secondly, APL patients formed a distinct subgroup, with more cells in G_0, fewer cells actively cycling and in $S/G_2/M$, and a greater proportion of cells likely to be in a G_1-arrested state than non-APL patients. They also had a lower geminin/MIB-1 ratio, implying a slower cell cycle time. This data is in accord with the fact that most of the cells in APL are differentiated to a relatively late maturational stage, by which time the proliferative potential is markedly reduced. No differences were observed between other FAB groups. There was also a significantly lower cleaved caspase LI in APL compared to other AML FAB types, which is in accord with the observation that high apoptosis rates are usually associated with the most rapidly proliferative tumours.

Thirdly, although cytogenetic risk group did not seem to have an impact on the expression of DNA replication licensing proteins and the derived cell cycle status, molecular genotype did. Cells from $NPM1^{MUT}$ AML were cycling more quickly, and this accords with the fact that *NPM1* mutations are associated with a higher presenting WCC (Dohner *et al*, 2005; Gale *et al*, 2008). Of note, although there was a higher proportion of cells licensed for replication, a similar relationship of a greater rate of cell cycling was not observed for cells from $FLT3^{ITD}$ AML, which is counterintuitive in that an activating mutation in a growth factor receptor would be expected to induce more rapid proliferation. It is possible that the major impact of a $FLT3^{ITD}$ is on the more primitive leukaemic progenitor cells and not the bulk leukaemic cells, but studies by Behbehani and colleagues have suggested that the more primitive AML progenitors in

FLT3^{ITD}-positive AML have a lower proliferative fraction than the same phenotypic subpopulation in other forms of AML (Behbehani *et al*, 2015).

Results were also presented showing that, compared to haematologically normal BMTs, the BMTs from AML patients had a similar proportion of cells licensed for replication but fewer actively cycling cells. However, those cells that were cycling were doing so at a greater speed. These results are complicated by the fact that there may be residual normal marrow in the AML patients, although it has been reported that this residual "normal marrow" does not behave normally in that it is functionally inhibited by the leukaemia cells (Miraki-Moud *et al*, 2013).

The results indicate that there are some major similarities and differences between AML and other types of malignancies that have been characterised. The fact that only a minority of bone marrow cells in the AML patients were in G_0 (median MCM2 LI for myeloid cells in the marrow was 81%) is similar to a number of other malignancies where this parameter has been assessed (Dudderidge *et al*, 2005a; Obermann *et al*, 2005b; Kulkarni *et al*, 2007; Kayes *et al*, 2009; Loddo *et al*, 2009). There was also a relatively high proportion of actively cycling cells (approximately one-third of myeloid cells), similarly comparable to various other malignancies, although considerably less than in Burkitt's lymphoma where nearly all the cells are actively cycling (Obermann *et al*, 2005b). However, a striking feature of the current findings is that those AML cells that were actively cycling were doing so rapidly (median geminin/MIB-1 ratio, 0.51). This is about twice the value observed in ovarian and penile cancer, and nearly three times that in breast cancer (Kulkarni *et al*, 2007; Kayes *et al*, 2009; Loddo *et al*, 2009), and this speed of cycling may therefore contribute to the observed sensitivity of AML cells to the cell cycle phase-specific agents that have been the backbone of standard therapy for several decades.

The considerable heterogeneity in the cell cycle profile observed between patient samples also highlights a number of important factors. In spite of the general sensitivity of AML cells to cell cycle phase-specific agents, it was noteworthy that the proportion of actively cycling cells did not significantly correlate with the CR rate. Nevertheless, the speed of cell cycling, as assessed by the geminin/MIB-1 ratio, was inversely related to chemoresistance to Ara-C plus anthracycline combination chemotherapy, and

although it was most significant after the initial cycle of intensive induction therapy, it was still apparent when best response at any time was considered. The impact on CR rates was much less apparent than on chemoresistance, and this was largely due to the higher treatment-related mortality in those patients with the most rapidly proliferative disease. The latter have, on average, a much higher presenting WCC, and it has been shown previously that this is a major risk factor for treatment-related mortality (Walter *et al*, 2011; Othus *et al*, 2014).

The rate of relapse was significantly higher in the quartile with the slowest cell cycling time than in the other quartiles, although this did not translate into a significant difference in survival, which was again likely to be due in part to the confounding effect of the increased treatment-related deaths in those with the fastest cell cycling time. This finding has interesting biological implications. Although the attainment of CR is a reflection of the properties of the bulk of the leukaemic cells, relapse is thought to originate from the leukaemic stem cells (LSC), which have a more primitive phenotype and are thought to proliferate more slowly than the bulk of cells. Nonetheless, the data suggests that the study of proliferation kinetics in the bulk population may be a surrogate for the chemosensitivity of the LSCs, probably indicating that, in those cases where the bulk cells are proliferating more quickly, so too are the LSCs, albeit at a lower level than in the bulk population. This may be testable by multi-parameter immunophenotypic analysis of AML progenitor cell populations using flow cytometry or mass cytometry on single cell suspensions made from BMTs.

An interesting question is whether a greater proportion of cells in $S/G_2/M$ really does reflect an increased speed of cycling or, perhaps, may indicate a longer time to go through these phases of the cycle. The studies presented here cannot provide direct information on this question, but the fact that the derived speed of cycling correlated with the presenting white cell count supports this assertion and, in addition, it reflects the time spent by the leukaemic cells at a vulnerable point of the cell cycle and appears to correlate with chemosensitivity.

A caveat to interpreting the data presented here is the heterogeneity inherent within a population of leukaemic cells. All studies of this kind are an attempt to average the behaviour of cells. In addition, since the start of this study, several new mutations have

been discovered in AML that may have bearing on cell cycle dynamics. Although *FLT3*^{ITD} and *NPM1*^{MUT} are powerful determinants of outcome, and *NPM1*^{MUT} are associated with distinctive features that appear to trump other lesions, it is possible that other coincidental mutations may have a strong influence on cell cycle dynamics, response to chemotherapy, and survival. For example, within the *NPM1*^{MUT} cohort there appeared to be a bimodal distribution of values in the geminin/MIB-1 ratios (Figure 5.9B) that may relate to other associated mutations such as in the *DNMT3A* gene. Ultimately, it would be desirable to fully annotate the cohort in terms of genetic mutations and also to see if these results could be replicated in a validation cohort.

From a clinical perspective, being able to predict the likelihood of chemo-resistance to the first cycle of therapy is of limited value as results based on trephine biopsies are not immediately available before the initial treatment is given, although it is possible that trephine 'rolls' could be used to achieve an earlier cell cycle assessment. The ability to predict outcome after one cycle of treatment might, however, be of therapeutic value, and the landmark analysis from the time patients started their second cycle of chemotherapy suggests that a very poor prognosis group (those with the slowest cell cycling time) can be identified, regardless of whether or not CR has been attained. Such patients might benefit from a change of therapy to agents that are not cell cycle phase-specific.

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

In addition to impaired differentiation, AML is a disease marked by dysregulation of the cell cycle, and at least part of the efficacy of the main chemotherapy agents used for intensive induction therapy in patients with the disease stems from their ability to target actively cycling cells. In addition, many of the new agents currently in clinical trials target key cell cycle proteins. As such, a full understanding of the heterogeneous responses to established and newer treatments requires knowledge of cell cycle dynamics in AML, as well as an appreciation of how this relates to established biological and prognostic factors, including cytogenetic and molecular lesions. Despite several decades of research examining the cell cycle in AML, the reports on outcome according to cell cycle dynamics are inconsistent, and in many cases are contradictory. Moreover, there are few studies that have investigated the relationship between the cell cycle and cytogenetics, and no published studies that have addressed the relationship between recurrent molecular mutations, the cell cycle, and outcome. Importantly, all previous studies used cells taken from BMA and/or PB for the cell cycle analysis.

This thesis set out to investigate whether the examination of cell cycle licensing proteins in bone marrow biopsies might give a more accurate assessment of cell cycle status in diagnostic samples from patients with AML. It then sought to correlate cell cycle status assessed using this method with biological and clinical features of disease.

6.1 Summary of results

The initial studies in this thesis sought to assess whether the expression of replication licensing proteins in blasts from patients with AML differed according to whether the source of the blasts was BMT, BMA, or PB. IHC and ICC assessing MCM2, MIB-1, and geminin was used to compare the cell cycle status between blasts sampled in parallel from these tissue compartments from 8 patients with either a new diagnosis or relapsed disease prior to treatment. An important initial finding was that a similar

number of blasts from PB, BMA, and BMT samples were in a non-G_0 state and "in cycle" (MCM2 positive), but that the number of cells actively progressing through the cell cycle or that had progressed beyond G_1 (MIB-1 and geminin positive respectively) was markedly different between the blasts from each source. Although there was a correlation between the number of cells actively cycling or progressing beyond G_1, as defined by the expression of these proteins in each compartment, the correlation was weak. Importantly, derived values such as the number of blasts that had progressed beyond G_1 as a proportion of actively cycling cells (the geminin/MIB-1 ratio) were not correlated, and were likely to be particularly inaccurate where the number of MIB-1 positive cells was low because small unit changes could result in apparently large changes when expressed as a percentage. The corollary of this is that the published studies that have used BMA or PB samples to measure the cell cycle have not, in fact, captured the true cell cycle dynamics within the bone marrow. This may account for some of the large variation in results seen in previous studies. One likely contributor to this inaccuracy is that, as demonstrated in the studies presented here, blasts from PB but not BMT samples were in an arrested G_1 state and therefore the presence of PB cells "contaminating" BMA samples, particularly likely when large volumes of marrow are sampled, would influence the results obtained. Further studies from our group that determined the volumetric contribution of BMT and PB to BMA in matched samples from 13 individuals with a morphologically normal bone marrow found that a median 53% of the BMA cells were BM-derived and 47% were PB-derived (Sellar *et al*, 2016). As these relative proportions are dependent on the absolute cell concentrations in the samples, the analysis also predicted that, in leukaemic samples, the contribution from PB cells would be greatest when the PB WCC is elevated (Sellar *et al*, 2016). This was supported by data presented in chapter 5 demonstrating that higher levels of geminin and geminin/MIB-1 positive cells in BMT samples correlated with a higher presenting WCC. Therefore analysis of BMA and PB samples may underestimate the level of proliferation in many of those patients in whom the proliferative drive is greatest. This implies that for accurate cell cycle analysis, BMT samples must be used.

The discrepancies observed between the results using blasts from BMT samples and those from either BMA or PB samples were verified using two other methods: flow

cytometry and gene expression profiling using RNAseq data. Formalin fixed cells from the same 8 patients were permeabilised and propidium iodide used to determine the DNA content of individual cells and hence their cell cycle status. The summation of the cell cycle status of cells in these patients were both highly correlated, and showed good agreement with, the results obtained by IHC and ICC further demonstrating that using blasts from BMA or PB underestimates the number of actively cycling cells and cells beyond G_1 within the bone marrow. In a further patient with AML and >90% blasts in samples from BMT, BMA, and PB RNA was extracted for comparative gene expression profiling by RNAseq. Many of the key DNA replication licensing genes previously described were shown to be downregulated in PB and BMA compared to BMT including geminin and members of the MCM complex. In addition, many of the cell cycle targets of new therapeutics were downregulated compared to BMT including polo-like kinases and aurora kinases. Studies also identified a number of genes that were differentially expressed between marrow and blood that were not primarily related to the cell cycle. Although this represents data from a single patient, two of these genes, *ATF3* and *FOSB*, are transcription factors involved in the stress response and normal development that a previous study had found to be differentially expressed in blasts from BMA and PB samples (Cheung *et al*, 2009). This suggests that the transcriptional profile of blasts in marrow does indeed differ from that of blasts in the peripheral blood. Another differentially expressed transcription factor, *JUN,* is increasingly recognised as being important to fundamental aspects of disease biology, including the unfolded protein response (Zhou *et al*, 2016). It would be interesting to verify these differences in further patients as well as investigate their biological relevance, in particular whether this can be exploited therapeutically.

These differences also have implications for the conclusions drawn in published studies of gene expression analysis in samples from patients with AML as they have used BMA or PB blasts to define gene expression signatures. An analysis assessing the prognostic signature described by (Bullinger *et al*, 2004) showed that in the matched samples studied here, 34% of genes in this signature had either a 2-fold lower expression or no expression in the PB or BMA samples as compared to the BMT sample. Indeed, many of the genes they identified as prognostic (e.g. *COL1A1*, the major component of type 1 collagen) do not have an obvious relationship to AML blasts and would be predicted to be most highly expressed in stromal tissue. Thus, the gene expression profile reported

may, in fact, not be reporting differences between blasts but rather reflect the level of haemodilution, the peripheral blood WCC, or indeed the amount of stromal tissue aspirated. These could all be influenced by multiple factors, for example, the relationship between blasts and the stroma, or the level of fibrosis in the patient.

Having identified that an accurate representation of cell cycle dynamics in the bone marrow of patients with AML can only be obtained using biopsy samples, chapter 4 described the identification and characterisation of a cohort of 181 patients with diagnostic biopsies available for further cell cycle analysis. This cohort was defined for major clinical features including age, presenting WCC, and cytogenetics, as well as clinical outcome according to treatment received, achievement of CR, CIR, and OS. Another key component in defining this cohort was the identification of archived marrow aspirates that were used as a source of DNA for the analysis of *FLT3*-ITD and *NPM1* mutations. Crucially, the incidence and clinical impact of established risk factors such as cytogenetic and molecular lesions were broadly in keeping with large published cohorts, which indicated that this AML cohort was suitable for the assessment of other potential prognostic factors.

Biopsies from this cohort of 181 patients were then assessed by IHC for expression of the cell cycle proteins MCM2, MKI67, and geminin (chapter 5). This analysis was aided by an automated digital analysis system that was validated as part of the study. The results showed that cell cycle dynamics were highly heterogeneous between patients. Proliferative drive was lowest in those patients with APL, but was otherwise not related to the state of differentiation as denoted by the FAB category of patients. Increased proliferation, as assessed by the geminin LI or geminin/MIB-1 ratio, was seen in patients with *NPM1* mutations but not those with a *FLT3*-ITD. Crucially, these values were also higher in those patients achieving CR following one cycle of intensive induction therapy, thereby providing a possible link between the increased remission rates and early responses to treatment seen in patients with *NPM1* mutations (Schneider et al, 2009). In a multivariate analysis, the association of higher geminin LI or geminin/MIB-1 ratio with achievement of CR was maintained. Higher geminin and geminin/MIB-1 ratios were also associated with death during first induction therapy, although the reasons for this are unclear. It is also noteworthy that the CIR was greatest in those patients with the lowest geminin/MIB-1 ratio, suggesting that these patients

may have a greater number of cells that can escape the effects of chemotherapy and serve as a source of relapse. Although this study did not assess the source of relapse in terms of quiescent stem cells or resistant subclones, it does nevertheless suggest that the analysis of the bulk leukaemia cell population can inform on biology and clinically relevant endpoints.

6.2 Future studies

The results of the studies presented here indicate numerous potential areas for further investigation. Firstly, the relationship between cell-cycle licensing proteins, molecular subtype, and response to induction therapy needs to be verified in an independent cohort of patients. Ideally, such a cohort would have information on patients' performance status that had been prospectively collected. This is key information with prognostic implications, for example including death from induction therapy, which is missing from the cohort studied here.

A more extensive retrospective analysis of samples from patients entered into clinical trials might also assist in selecting patients that are more likely to respond to specific therapies. For example, in studies of increased anthracycline dose in induction therapy, might improvements be restricted to or more pronounced in particular biological subgroups as defined by cell cycle status? Or in trials employing G-CSF in an attempt to stimulate cells into cycle, might the effect only be seen in those patients with few cells already committed to cycle rather than in those with active and rapidly cycling cells where no additional benefit can be gained? This is clearly speculative but hints at the sort of questions that could be investigated to give further context to the results obtained from past clinical trials.

A key finding from this thesis that warrants further investigation is the increased numbers of geminin-positive cells and increased geminin/MIB-1 ratio in the group of patients with *NPM1* mutations. Although these values were elevated in the *NPM1* mutant cohort as a whole, they were highly variable. This suggests intriguing and

potentially key differences in disease biology within this group, and is important as the presence of an *NPM1* mutation is generally considered to be a good risk factor. All three patients with an *NPM1* mutation that failed to attain remission with one cycle of chemotherapy were in the group with the lowest geminin/MIB-1 ratios. However, because of the limited number of patients failing to achieve remission in this genotype group, it is not clear that this reflects a true difference in response to induction therapy, and expanding the number of patients investigated might help to address this issue. It would also be interesting to determine whether differences in cell cycle dynamics exist according to the type of *NPM1* mutation (e.g. the most common type A mutant versus non-A mutants) to help discern whether the functional consequences of these mutations differ, especially as recently presented data suggests that their impact on outcome might vary (Selim *et al*, 2016).

To further refine the relationship between cell cycle licensing proteins and response to treatment, it will be important to define the landscape of mutations and their co-incidence beyond *NPM1* and *FLT3*-ITD. Chapter 1 described the varied genetic landscape of AML, and many of these mutations have been described since this thesis project was first conceived. The key mutations present in an individual patient can now be rapidly and relatively inexpensively defined using technology that is available as part of routine diagnostics. For example, the "Rapid Heme Panel" employed at the Dana-Farber Cancer Center can identify mutations in 95 different genes within 72 hours of disease presentation. Given the number of mutation combinations that are possible, the detection of differences would require a significant expansion of the patient cohort studied. Another approach would be to investigate the mechanism underlying specific genetic lesions, their impact on cell cycle status, and the response to treatment using animal models of disease that can examine combinations of lesions. As an example, up to five genes have been modified in a single mouse haematopoietic stem cell using the CRISPR-Cas9 gene editing system. (Heckl *et al*, 2014).

The RNA-seq data presented here was limited to matched samples from a single patient. It would therefore be interesting to investigate gene expression profiles using such data from multiple BMT samples in order to determine whether this could lead to a more robust prognostic scoring system. Although the modelling of the stromal signature would be a complicating factor, a similar issue has been overcome in solid tumours

where the non-malignant tissue forms a sizeable proportion of the sample. Furthermore, evaluation of the stromal signature in AML patients may prove useful, especially as there is an increasing body of knowledge supporting the role of non-haematopoietic cells in the bone marrow in both the maintenance and initiation of leukaemia (Kode *et al*, 2014; Krause & Scadden, 2015). Defining a combined leukaemia and stromal signature associated with disease resistance could lead to a major advancement in understanding disease biology.

Ultimately, the aim of a better appreciation of the cell cycle dynamics is to improve patient therapy by defining subgroups of those who are likely, or not likely, to benefit from treatment targeting cell cycle proteins. In addition to the agents highlighted in chapter 1 that are currently being evaluated in clinical trials, there are two newer approaches to targeting the cell cycle in G_1-S phase that are in pre-clinical development.

One approach targets the non-redundant kinase CDC7 that phosphorylates MCM2, MCM4, and MCM6, and serves as a key regulator of G_1-S progression (Figure 1.2). In theory, inhibiting CDC7 would cause normal cells to pause prior to S phase whereas cells with an abnormal G_1-S phase checkpoint, particularly those with an abnormal TP53 pathway, would progress into S phase. As CDC7 is required to activate the pre-replication complex, these abnormal cells would enter S phase with an insufficient number of active replication forks and progress into a catastrophic mitosis (Montagnoli *et al*, 2008; Vanotti *et al*, 2008; Tudzarova *et al*, 2010). There is some evidence from RNA interference experiments and inhibition using novel small molecules that this may indeed be selectively active against malignant cells (Kulkarni *et al*, 2009; Rodriguez-Acebes *et al*, 2010; Huggett *et al*, 2016). To further understand the role of CDC7, in work performed since the completion of these studies, I have started to investigate the expression of CDC7 and association with genetic and clinical features in the defined cohort of patients. In addition, I have been investigating therapeutic potential and selectivity of CDC7 inhibition using an inducible shRNA system and a small molecule inhibitor XL413 (Koltun *et al*, 2012; Sasi *et al*, 2014) in cell lines, primary AML samples, and normal CD34+ cells.

Another approach is targeting GSPT1 (eRF3a), a translation termination factor that binds eRF1 and in doing so mediates stop codon recognition and nascent protein release

from ribosomes. Depletion of GSPT1 has previously been shown to cause G_1 arrest (Chauvin *et al*, 2007). A recently published study of a novel compound related to lenalidomide that is able to selectively target GSPT1 for ubiquitination and degradation has shown that it has potent anti-tumour activity in both established AML cell lines and samples from patients (Matyskiela *et al*, 2016). Defining the conditions through which AML and the cell cycle can be targeted by this approach is a major component of my current fellowship in the laboratory of Dr Benjamin Ebert at the Brigham and Women's Hospital and the Broad Institute of Harvard/MIT in Boston MA, USA.

6.3 Conclusions

In conclusion, this thesis has described the assessment of cell cycle licensing protein expression in bone marrow biopsies of patients with AML. It has demonstrated that this method better reflects the cell cycle dynamics of the disease in marrow and is the first study to define the stages of the cell cycle in AML exclusively using biopsies. In addition these studies have described a link between the expression of geminin and the geminin/MIB-1 ratio and response to therapy, in particular in patients with *NPM1* mutations. As such, this work contributes to the current understanding of the biology of AML, and provides a rationale for the future analysis of patient cell cycle status as an adjunct to developing personalised therapeutic strategies.

PUBLICATIONS ARISING FROM WORK IN THIS THESIS

Sellar, R.S., Fraser, L., Khwaja, A., Gale, R.E., Marafioti, T., Akarca, A., Hubank, M., Brooks, T., Stoeber, K., Williams, G. & Linch, D.C. (2016) Cell cycle status in AML blast cells from peripheral blood, bone marrow aspirates and trephines and implications for biological studies and treatment. *British journal of haematology,* **174,** 275-279.

REFERENCES

Abbas, S., Lugthart, S., Kavelaars, F.G., Schelen, A., Koenders, J.E., Zeilemaker, A., van Putten, W.J., Rijneveld, A.W., Lowenberg, B. & Valk, P.J. (2010) Acquired mutations in the genes encoding IDH1 and IDH2 both are recurrent aberrations in acute myeloid leukemia: prevalence and prognostic value. *Blood,* **116,** 2122-2126.

Abu-Duhier, F.M., Goodeve, A.C., Wilson, G.A., Gari, M.A., Peake, I.R., Rees, D.C., Vandenberghe, E.A., Winship, P.R. & Reilly, J.T. (2000) FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. *British journal of haematology,* **111,** 190-195.

Abu-Duhier, F.M., Goodeve, A.C., Wilson, G.A., Care, R.S., Peake, I.R. & Reilly, J.T. (2001) Genomic structure of human FLT3: implications for mutational analysis. *British journal of haematology,* **113,** 1076-1077.

Aggarwal, P., Lessie, M.D., Lin, D.I., Pontano, L., Gladden, A.B., Nuskey, B., Goradia, A., Wasik, M.A., Klein-Szanto, A.J., Rustgi, A.K., Bassing, C.H. & Diehl, J.A. (2007) Nuclear accumulation of cyclin D1 during S phase inhibits Cul4-dependent Cdt1 proteolysis and triggers p53-dependent DNA rereplication. *Genes Dev,* **21,** 2908-2922.

Alexandrov, L.B., Nik-Zainal, S., Wedge, D.C., Aparicio, S.A., Behjati, S., Biankin, A.V., Bignell, G.R., Bolli, N., Borg, A., Borresen-Dale, A.L., Boyault, S., Burkhardt, B., Butler, A.P., Caldas, C., Davies, H.R., Desmedt, C., Eils, R., Eyfjord, J.E., Foekens, J.A., Greaves, M., Hosoda, F., Hutter, B., Ilicic, T., Imbeaud, S., Imielinski, M., Jager, N., Jones, D.T., Jones, D., Knappskog, S., Kool, M., Lakhani, S.R., Lopez-Otin, C., Martin, S., Munshi, N.C., Nakamura, H., Northcott, P.A., Pajic, M., Papaemmanuil, E., Paradiso, A., Pearson, J.V., Puente, X.S., Raine, K., Ramakrishna, M., Richardson, A.L., Richter, J., Rosenstiel, P., Schlesner, M., Schumacher, T.N., Span, P.N., Teague, J.W., Totoki, Y., Tutt, A.N., Valdes-Mas, R., van Buuren, M.M., van 't Veer, L., Vincent-Salomon, A., Waddell, N., Yates, L.R., Australian Pancreatic Cancer Genome, I., Consortium, I.B.C., Consortium, I.M.-S., PedBrain, I., Zucman-Rossi, J., Futreal, P.A., McDermott, U., Lichter, P., Meyerson, M., Grimmond, S.M., Siebert, R., Campo, E., Shibata, T., Pfister, S.M., Campbell, P.J. & Stratton, M.R. (2013) Signatures of mutational processes in human cancer. *Nature,* **500,** 415-421.

Allaudeen, H.S., Kozarich, J.W. & Sartorelli, A.C. (1982) Comparative effects of the 5'-triphosphates of 9-beta-(2'-azido-2'-deoxy-D-arabinofuranosyl)adenine and 9-beta-D-arabinofuranosyladenine on DNA polymerases from L1210 leukemia cells. *Nucleic Acids Res,* **10,** 1379-1387.

Allen, C., Hills, R.K., Lamb, K., Evans, C., Tinsley, S., Sellar, R., O'Brien, M., Yin, J.L., Burnett, A.K., Linch, D.C. & Gale, R.E. (2013) The importance of relative mutant level for evaluating impact on outcome of KIT, FLT3 and CBL mutations in core-binding factor acute myeloid leukemia. *Leukemia,* **27,** 1891-1901.

Amadori, S., Petti, M.C., Mastrovincenzo, C., Testa, M.G., Antonelli, A. & Mandelli, F. (1980) Blast cell kinetics and prognosis in acute nonlymphocytic leukemia. *Leukemia research,* **4,** 239-244.

Andersson, A., Johansson, B., Lassen, C., Mitelman, F., Billstrom, R. & Fioretos, T. (2004) Clinical impact of internal tandem duplications and activating point mutations in FLT3 in acute myeloid leukemia in elderly patients. *Eur J Haematol,* **72,** 307-313.

Anglana, M., Apiou, F., Bensimon, A. & Debatisse, M. (2003) Dynamics of DNA replication in mammalian somatic cells: nucleotide pool modulates origin choice and interorigin spacing. *Cell,* **114,** 385-394.

Appelbaum, F.R., Gundacker, H., Head, D.R., Slovak, M.L., Willman, C.L., Godwin, J.E., Anderson, J.E. & Petersdorf, S.H. (2006) Age and acute myeloid leukemia. *Blood,* **107,** 3481-3485.

Arber, D.A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M.J., Le Beau, M.M., Bloomfield, C.D., Cazzola, M. & Vardiman, J.W. (2016) The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood,* **127,** 2391-2405.

Arentson, E., Faloon, P., Seo, J., Moon, E., Studts, J.M., Fremont, D.H. & Choi, K. (2002) Oncogenic potential of the DNA replication licensing protein CDT1. *Oncogene,* **21,** 1150-1158.

Argiropoulos, B., Yung, E., Xiang, P., Lo, C.Y., Kuchenbauer, F., Palmqvist, L., Reindl, C., Heuser, M., Sekulovic, S., Rosten, P., Muranyi, A., Goh, S.L., Featherstone, M. & Humphries, R.K. (2010) Linkage of the potent leukemogenic activity of Meis1 to cell-cycle entry and transcriptional regulation of cyclin D3. *Blood,* **115,** 4071-4082.

Arias, E.E. & Walter, J.C. (2006) PCNA functions as a molecular platform to trigger Cdt1 destruction and prevent re-replication. *Nat Cell Biol,* **8,** 84-90.

Ayaru, L., Stoeber, K., Webster, G.J., Hatfield, A.R., Wollenschlaeger, A., Okoturo, O., Rashid, M., Williams, G. & Pereira, S.P. (2008) Diagnosis of pancreaticobiliary malignancy by detection of minichromosome maintenance protein 5 in bile aspirates. *Br J Cancer,* **98,** 1548-1554.

Bacher, U., Haferlach, C., Kern, W., Haferlach, T. & Schnittger, S. (2008) Prognostic relevance of FLT3-TKD mutations in AML: the combination matters--an analysis of 3082 patients. *Blood,* **111,** 2527-2537.

Bagley, B.N., Keane, T.M., Maklakova, V.I., Marshall, J.G., Lester, R.A., Cancel, M.M., Paulsen, A.R., Bendzick, L.E., Been, R.A., Kogan, S.C., Cormier, R.T., Kendziorski, C., Adams, D.J. & Collier, L.S. (2012) A dominantly acting murine allele of Mcm4 causes chromosomal abnormalities and promotes tumorigenesis. *PLoS Genet,* **8,** e1003034.

Bailis, J.M., Luche, D.D., Hunter, T. & Forsburg, S.L. (2008) Minichromosome maintenance proteins interact with checkpoint and recombination proteins to promote s-phase genome stability. *Mol Cell Biol,* **28,** 1724-1738.

Becker, H., Marcucci, G., Maharry, K., Radmacher, M.D., Mrozek, K., Margeson, D., Whitman, S.P., Wu, Y.Z., Schwind, S., Paschka, P., Powell, B.L., Carter, T.H., Kolitz, J.E., Wetzler, M., Carroll, A.J., Baer, M.R., Caligiuri, M.A., Larson, R.A. & Bloomfield, C.D. (2010) Favorable prognostic impact of NPM1 mutations in older patients with cytogenetically normal de novo acute myeloid leukemia and associated gene- and microRNA-expression signatures: a Cancer and Leukemia Group B study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **28,** 596-604.

Behbehani, G.K., Samusik, N., Bjornson, Z.B., Fantl, W.J., Medeiros, B.C. & Nolan, G.P. (2015) Mass Cytometric Functional Profiling of Acute Myeloid Leukemia Defines Cell-Cycle and Immunophenotypic Properties That Correlate with Known Responses to Therapy. *Cancer Discov,* **5,** 988-1003.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A., Gralnick, H.R. & Sultan, C. (1976) Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol,* **33,** 451-458.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A., Gralnick, H.R. & Sultan, C. (1985) Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med,* **103,** 620-625.

Bicknell, L.S., Bongers, E.M., Leitch, A., Brown, S., Schoots, J., Harley, M.E., Aftimos, S., Al-Aama, J.Y., Bober, M., Brown, P.A., van Bokhoven, H., Dean, J., Edrees, A.Y., Feingold, M., Fryer, A., Hoefsloot, L.H., Kau, N., Knoers, N.V., Mackenzie, J., Opitz, J.M., Sarda, P., Ross, A., Temple, I.K., Toutain, A., Wise, C.A., Wright, M. & Jackson, A.P. (2011a) Mutations in the pre-replication complex cause Meier-Gorlin syndrome. *Nat Genet,* **43,** 356-359.

Bicknell, L.S., Walker, S., Klingseisen, A., Stiff, T., Leitch, A., Kerzendorfer, C., Martin, C.A., Yeyati, P., Al Sanna, N., Bober, M., Johnson, D., Wise, C., Jackson, A.P., O'Driscoll,

M. & Jeggo, P.A. (2011b) Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause microcephalic primordial dwarfism resembling Meier-Gorlin syndrome. *Nat Genet,* **43,** 350-355.

Blow, J.J. & Dutta, A. (2005) Preventing re-replication of chromosomal DNA. *Nature reviews. Molecular cell biology,* **6,** 476-486.

Bochtler, T., Stolzel, F., Heilig, C.E., Kunz, C., Mohr, B., Jauch, A., Janssen, J.W., Kramer, M., Benner, A., Bornhauser, M., Ho, A.D., Ehninger, G., Schaich, M. & Kramer, A. (2013) Clonal heterogeneity as detected by metaphase karyotyping is an indicator of poor prognosis in acute myeloid leukemia. *J Clin Oncol,* **31,** 3898-3905.

Boissel, N., Cayuela, J.M., Preudhomme, C., Thomas, X., Grardel, N., Fund, X., Tigaud, I., Raffoux, E., Rousselot, P., Sigaux, F., Degos, L., Castaigne, S., Fenaux, P. & Dombret, H. (2002) Prognostic significance of FLT3 internal tandem repeat in patients with de novo acute myeloid leukemia treated with reinforced courses of chemotherapy. *Leukemia,* **16,** 1699-1704.

Boissel, N., Renneville, A., Biggio, V., Philippe, N., Thomas, X., Cayuela, J.M., Terre, C., Tigaud, I., Castaigne, S., Raffoux, E., De Botton, S., Fenaux, P., Dombret, H. & Preudhomme, C. (2005) Prevalence, clinical profile, and prognosis of NPM mutations in AML with normal karyotype. *Blood,* **106,** 3618-3620.

Boissel, N., Nibourel, O., Renneville, A., Gardin, C., Reman, O., Contentin, N., Bordessoule, D., Pautas, C., de Revel, T., Quesnel, B., Huchette, P., Philippe, N., Geffroy, S., Terre, C., Thomas, X., Castaigne, S., Dombret, H. & Preudhomme, C. (2010) Prognostic impact of isocitrate dehydrogenase enzyme isoforms 1 and 2 mutations in acute myeloid leukemia: a study by the Acute Leukemia French Association group. *J Clin Oncol,* **28,** 3717-3723.

Bolli, N., Payne, E.M., Grabher, C., Lee, J.S., Johnston, A.B., Falini, B., Kanki, J.P. & Look, A.T. (2010) Expression of the cytoplasmic NPM1 mutant (NPMc+) causes the expansion of hematopoietic cells in zebrafish. *Blood,* 115, 3329-3340.

Bonetti, P., Davoli, T., Sironi, C., Amati, B., Pelicci, P.G. & Colombo, E. (2008) Nucleophosmin and its AML-associated mutant regulate c-Myc turnover through Fbw7 gamma. *J Cell Biol,* **182,** 19-26.

Booth, D.G., Takagi, M., Sanchez-Pulido, L., Petfalski, E., Vargiu, G., Samejima, K., Imamoto, N., Ponting, C.P., Tollervey, D., Earnshaw, W.C. & Vagnarelli, P. (2014) Ki-67 is a PP1-interacting protein that organises the mitotic chromosome periphery. *Elife,* **3,** e01641.

Braess, J., Jahns-Streubel, G., Schoch, C., Haase, D., Haferlach, T., Fiegl, M., Voss, S., Kern, W., Schleyer, E. & Hiddemann, W. (2001) Proliferative activity of leukaemic blasts and cytosine arabinoside pharmacodynamics are associated with cytogenetically defined prognostic subgroups in acute myeloid leukaemia. *British journal of haematology,* **113,** 975-982.

Breems, D.A., Van Putten, W.L., Huijgens, P.C., Ossenkoppele, G.J., Verhoef, G.E., Verdonck, L.F., Vellenga, E., De Greef, G.E., Jacky, E., Van der Lelie, J., Boogaerts, M.A. & Lowenberg, B. (2005) Prognostic index for adult patients with acute myeloid leukemia in first relapse. *J Clin Oncol,* **23,** 1969-1978.

Breems, D.A., Van Putten, W.L., De Greef, G.E., Van Zelderen-Bhola, S.L., Gerssen-Schoorl, K.B., Mellink, C.H., Nieuwint, A., Jotterand, M., Hagemeijer, A., Beverloo, H.B. & Lowenberg, B. (2008) Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. *J Clin Oncol,* **26,** 4791-4797.

British Committee for Standards in, H., Milligan, D.W., Grimwade, D., Cullis, J.O., Bond, L., Swirsky, D., Craddock, C., Kell, J., Homewood, J., Campbell, K., McGinley, S., Wheatley, K. & Jackson, G. (2006) Guidelines on the management of acute myeloid leukaemia in adults. *British journal of haematology,* **135,** 450-474.

Brown, P., McIntyre, E., Rau, R., Meshinchi, S., Lacayo, N., Dahl, G., Alonzo, T.A., Chang, M., Arceci, R.J. & Small, D. (2007) The incidence and clinical significance of nucleophosmin mutations in childhood AML. *Blood,* **110,** 979-985.

Buchner, T., Berdel, W.E., Haferlach, C., Haferlach, T., Schnittger, S., Muller-Tidow, C., Braess, J., Spiekermann, K., Kienast, J., Staib, P., Gruneisen, A., Kern, W., Reichle, A., Maschmeyer, G., Aul, C., Lengfelder, E., Sauerland, M.C., Heinecke, A., Wormann, B. & Hiddemann, W. (2009) Age-related risk profile and chemotherapy dose response in acute myeloid leukemia: a study by the German Acute Myeloid Leukemia Cooperative Group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **27,** 61-69.

Bullinger, L., Dohner, K., Bair, E., Frohling, S., Schlenk, R.F., Tibshirani, R., Dohner, H. & Pollack, J.R. (2004) Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *The New England journal of medicine,* **350,** 1605-1616.

Burnett, A.K., Milligan, D., Prentice, A.G., Goldstone, A.H., McMullin, M.F., Hills, R.K. & Wheatley, K. (2007) A comparison of low-dose cytarabine and hydroxyurea with or without all-trans retinoic acid for acute myeloid leukemia and high-risk myelodysplastic syndrome in patients not considered fit for intensive treatment. *Cancer,* **109,** 1114-1124.

Burnett, A.K., Hills, R.K., Milligan, D., Kjeldsen, L., Kell, J., Russell, N.H., Yin, J.A., Hunter, A., Goldstone, A.H. & Wheatley, K. (2011) Identification of patients with acute myeloblastic leukemia who benefit from the addition of gemtuzumab ozogamicin: results of the MRC AML15 trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **29,** 369-377.

Burnett, A.K., Russell, N.H., Hills, R.K., Hunter, A.E., Kjeldsen, L., Yin, J., Gibson, B.E., Wheatley, K. & Milligan, D. (2013) Optimization of chemotherapy for younger patients with acute myeloid leukemia: results of the medical research council AML15 trial. *J Clin Oncol,* **31,** 3360-3368.

Burnett, A.K., Russell, N., Hills, R.K., Panoskaltsis, N., Khwaja, A., Hemmaway, C., Cahalin, P., Clark, R.E. & Milligan, D. (2015a) A randomised comparison of the novel nucleoside analogue sapacitabine with low-dose cytarabine in older patients with acute myeloid leukaemia. *Leukemia,* **29,** 1312-1319.

Burnett, A.K., Russell, N.H., Hills, R.K., Bowen, D., Kell, J., Knapper, S., Morgan, Y.G., Lok, J., Grech, A., Jones, G., Khwaja, A., Friis, L., McMullin, M.F., Hunter, A., Clark, R.E., Grimwade, D. & Group, U.K.N.C.R.I.A.M.L.W. (2015b) Arsenic trioxide and all-trans retinoic acid treatment for acute promyelocytic leukaemia in all risk groups (AML17): results of a randomised, controlled, phase 3 trial. *Lancet Oncol,* **16,** 1295-1305.

Burnett, A.K., Russell, N.H., Hills, R.K. & United Kingdom National Cancer Research Institute Acute Myeloid Leukemia Study, G. (2016) Higher daunorubicin exposure benefits FLT3 mutated acute myeloid leukemia. *Blood,* **128,** 449-452.

Busque, L., Mio, R., Mattioli, J., Brais, E., Blais, N., Lalonde, Y., Maragh, M. & Gilliland, D.G. (1996) Nonrandom X-inactivation patterns in normal females: lyonization ratios vary with age. *Blood,* **88,** 59-65.

Cancer Genome Atlas Research, N. (2013) Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med,* **368,** 2059-2074.

Casey, J.P., Nobbs, M., McGettigan, P., Lynch, S. & Ennis, S. (2012) Recessive mutations in MCM4/PRKDC cause a novel syndrome involving a primary immunodeficiency and a disorder of DNA repair. *J Med Genet,* **49,** 242-245.

Cazzaniga, G., Dell'Oro, M.G., Mecucci, C., Giarin, E., Masetti, R., Rossi, V., Locatelli, F., Martelli, M.F., Basso, G., Pession, A., Biondi, A. & Falini, B. (2005) Nucleophosmin mutations in childhood acute myelogenous leukemia with normal karyotype. *Blood,* **106,** 1419-1422.

Chauvin, C., Salhi, S. & Jean-Jean, O. (2007) Human eukaryotic release factor 3a depletion causes cell cycle arrest at G1 phase through inhibition of the mTOR pathway. *Molecular and cellular biology,* **27,** 5619-5629.

Chen, X., Xie, H., Wood, B.L., Walter, R.B., Pagel, J.M., Becker, P.S., Sandhu, V.K., Abkowitz, J.L., Appelbaum, F.R. & Estey, E.H. (2015) Relation of clinical response

and minimal residual disease and their prognostic impact on outcome in acute myeloid leukemia. *J Clin Oncol,* **33,** 1258-1264.

Cheng, K., Grisendi, S., Clohessy, J.G., Majid, S., Bernardi, R., Sportoletti, P. & Pandolfi, P.P. (2007) The leukemia-associated cytoplasmic nucleophosmin mutant is an oncogene with paradoxical functions: Arf inactivation and induction of cellular senescence. *Oncogene,* **26,** 7391-7400.

Cheng, K., Sportoletti, P., Ito, K., Clohessy, J.G., Teruya-Feldstein, J., Kutok, J.L. & Pandolfi, P.P. (2010) The cytoplasmic NPM mutant induces myeloproliferation in a transgenic mouse model. *Blood,* **115,** 3341-3345.

Cheung, A.M., Chow, H.C., Liang, R. & Leung, A.Y. (2009) A comparative study of bone marrow and peripheral blood CD34+ myeloblasts in acute myeloid leukaemia. *British journal of haematology,* **144,** 484-491.

Chong, J.P., Mahbubani, H.M., Khoo, C.Y. & Blow, J.J. (1995) Purification of an MCM-containing complex as a component of the DNA replication licensing system. *Nature,* **375,** 418-421.

Chou, W.C., Chou, S.C., Liu, C.Y., Chen, C.Y., Hou, H.A., Kuo, Y.Y., Lee, M.C., Ko, B.S., Tang, J.L., Yao, M., Tsay, W., Wu, S.J., Huang, S.Y., Hsu, S.C., Chen, Y.C., Chang, Y.C., Kuo, K.T., Lee, F.Y., Liu, M.C., Liu, C.W., Tseng, M.H., Huang, C.F. & Tien, H.F. (2011) TET2 mutation is an unfavorable prognostic factor in acute myeloid leukemia patients with intermediate-risk cytogenetics. *Blood,* **118,** 3803-3810.

Clay-Farrace, L., Pelizon, C., Santamaria, D., Pines, J. & Laskey, R.A. (2003) Human replication protein Cdc6 prevents mitosis through a checkpoint mechanism that implicates Chk1. *EMBO J,* **22,** 704-712.

Colombo, E., Martinelli, P., Zamponi, R., Shing, D.C., Bonetti, P., Luzi, L., Volorio, S., Bernard, L., Pruneri, G., Alcalay, M. & Pelicci, P.G. (2006) Delocalization and destabilization of the Arf tumor suppressor by the leukemia-associated NPM mutant. *Cancer research,* **66,** 3044-3050.

Cortez, D., Glick, G. & Elledge, S.J. (2004) Minichromosome maintenance proteins are direct targets of the ATM and ATR checkpoint kinases. *Proc Natl Acad Sci U S A,* **101,** 10078-10083.

Daver, N., Cortes, J., Ravandi, F., Patel, K.P., Burger, J.A., Konopleva, M. & Kantarjian, H. (2015) Secondary mutations as mediators of resistance to targeted therapy in leukemia. *Blood,* **125,** 3236-3245.

Davies, R.J., Freeman, A., Morris, L.S., Bingham, S., Dilworth, S., Scott, I., Laskey, R.A., Miller, R. & Coleman, N. (2002) Analysis of minichromosome maintenance proteins as a novel method for detection of colorectal cancer in stool. *Lancet,* **359,** 1917-1919.

del Canizo, M.C., Brufau, A., Almeida, J., Galende, J., Garcia Marcos, M.A., Mota, A., Garcia, R., Fernandez Calvo, J., Ramos, F., Fisac, P., Orfao, A. & San Miguel, J.F. (1998) In vitro growth in acute myeloblastic leukaemia: relationship with other clinico-biological characteristics of the disease. *British journal of haematology,* **103,** 137-142.

den Besten, W., Kuo, M.L., Williams, R.T. & Sherr, C.J. (2005) Myeloid leukemia-associated nucleophosmin mutants perturb p53-dependent and independent activities of the Arf tumor suppressor protein. *Cell Cycle,* **4,** 1593-1598.

Deng, Y., Jiang, L., Wang, Y., Xi, Q., Zhong, J., Liu, J., Yang, S., Liu, R., Wang, J., Huang, M., Tang, C. & Su, M. (2016) High expression of CDC6 is associated with accelerated cell proliferation and poor prognosis of epithelial ovarian cancer. *Pathol Res Pract,* **212,** 239-246.

Derolf, A.R., Kristinsson, S.Y., Andersson, T.M., Landgren, O., Dickman, P.W. & Bjorkholm, M. (2009) Improved patient survival for acute myeloid leukemia: a population-based study of 9729 patients diagnosed in Sweden between 1973 and 2005. *Blood,* **113,** 3666-3672.

Deschler, B. & Lubbert, M. (2006) Acute myeloid leukemia: Epidemiology and etiology. *Cancer,* **107,** 2099-2107.

Dickson, G.J., Bustraan, S., Hills, R.K., Ali, A., Goldstone, A.H., Burnett, A.K., Linch, D.C. & Gale, R.E. (2016) The value of molecular stratification for CEBPA(DM) and

NPM1(MUT) FLT3(WT) genotypes in older patients with acute myeloid leukaemia. *British journal of haematology,* **172,** 573-580.

Dohner, K., Schlenk, R.F., Habdank, M., Scholl, C., Rucker, F.G., Corbacioglu, A., Bullinger, L., Frohling, S. & Dohner, H. (2005) Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood,* **106,** 3740-3746.

Dosik, G.M., Barlogie, B., Smith, T.L., Gehan, E.A., Keating, M.J., McCredie, K.B. & Freireich, E.J. (1980) Pretreatment flow cytometry of DNA content in adult acute leukemia. *Blood,* **55,** 474-482.

Dudderidge, T.J., Stoeber, K., Loddo, M., Atkinson, G., Fanshawe, T., Griffiths, D.F. & Williams, G.H. (2005a) Mcm2, Geminin, and KI67 define proliferative state and are prognostic markers in renal cell carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **11,** 2510-2517.

Dudderidge, T.J., Stoeber, K., Loddo, M., Atkinson, G., Fanshawe, T., Griffiths, D.F. & Williams, G.H. (2005b) Mcm2, Geminin, and KI67 define proliferative state and are prognostic markers in renal cell carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **11,** 2510-2517.

Dufour, A., Schneider, F., Metzeler, K.H., Hoster, E., Schneider, S., Zellmeier, E., Benthaus, T., Sauerland, M.C., Berdel, W.E., Buchner, T., Wormann, B., Braess, J., Hiddemann, W., Bohlander, S.K. & Spiekermann, K. (2010) Acute myeloid leukemia with biallelic CEBPA gene mutations and normal karyotype represents a distinct genetic entity associated with a favorable clinical outcome. *J Clin Oncol,* **28,** 570-577.

Ekholm-Reed, S., Mendez, J., Tedesco, D., Zetterberg, A., Stillman, B. & Reed, S.I. (2004a) Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *J Cell Biol,* **165,** 789-800.

Ekholm-Reed, S., Spruck, C.H., Sangfelt, O., van Drogen, F., Mueller-Holzner, E., Widschwendter, M., Zetterberg, A. & Reed, S.I. (2004b) Mutation of hCDC4 leads to cell cycle deregulation of cyclin E in cancer. *Cancer Res,* **64,** 795-800.

Erba, H.P. (2007) Prognostic factors in elderly patients with AML and the implications for treatment. *Hematology Am Soc Hematol Educ Program,* **2007,** 420-428.

Falini, B., Mecucci, C., Tiacci, E., Alcalay, M., Rosati, R., Pasqualucci, L., La Starza, R., Diverio, D., Colombo, E., Santucci, A., Bigerna, B., Pacini, R., Pucciarini, A., Liso, A., Vignetti, M., Fazi, P., Meani, N., Pettirossi, V., Saglio, G., Mandelli, F., Lo-Coco, F., Pelicci, P.G. & Martelli, M.F. (2005) Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *The New England journal of medicine,* **352,** 254-266.

Feng, D., Tu, Z., Wu, W. & Liang, C. (2003) Inhibiting the expression of DNA replication-initiation proteins induces apoptosis in human cancer cells. *Cancer Res,* **63,** 7356-7364.

Fernandez, H.F., Sun, Z., Yao, X., Litzow, M.R., Luger, S.M., Paietta, E.M., Racevskis, J., Dewald, G.W., Ketterling, R.P., Bennett, J.M., Rowe, J.M., Lazarus, H.M. & Tallman, M.S. (2009) Anthracycline dose intensification in acute myeloid leukemia. *The New England journal of medicine,* **361,** 1249-1259.

Ffrench, M., Bryon, P.A., Fiere, D., Vu Van, H., Gentilhomme, O., Adeleine, P. & Viala, J.J. (1985) Cell-cycle, protein content, and nuclear size in acute myeloid leukemia. *Cytometry,* **6,** 47-53.

Freeman, S.D., Virgo, P., Couzens, S., Grimwade, D., Russell, N., Hills, R.K. & Burnett, A.K. (2013) Prognostic relevance of treatment response measured by flow cytometric residual disease detection in older patients with acute myeloid leukemia. *J Clin Oncol,* **31,** 4123-4131.

Frohling, S., Schlenk, R.F., Breitruck, J., Benner, A., Kreitmeier, S., Tobis, K., Dohner, H., Dohner, K. & leukemia, A.M.L.S.G.U.A.m. (2002) Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. *Blood,* **100,** 4372-4380.

Gaidzik, V.I., Paschka, P., Spath, D., Habdank, M., Kohne, C.H., Germing, U., von Lilienfeld-Toal, M., Held, G., Horst, H.A., Haase, D., Bentz, M., Gotze, K., Dohner, H., Schlenk, R.F., Bullinger, L. & Dohner, K. (2012) TET2 mutations in acute myeloid leukemia (AML): results from a comprehensive genetic and clinical analysis of the AML study group. *J Clin Oncol,* **30,** 1350-1357.

Gale, R.E., Fielding, A.K., Harrison, C.N. & Linch, D.C. (1997) Acquired skewing of X-chromosome inactivation patterns in myeloid cells of the elderly suggests stochastic clonal loss with age. *British journal of haematology,* **98,** 512-519.

Gale, R.E., Green, C., Allen, C., Mead, A.J., Burnett, A.K., Hills, R.K. & Linch, D.C. (2008) The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. *Blood,* **111,** 2776-2784.

Gale, R.E., Lamb, K., Allen, C., El-Sharkawi, D., Stowe, C., Jenkinson, S., Tinsley, S., Dickson, G., Burnett, A.K., Hills, R.K. & Linch, D.C. (2015) Simpson's Paradox and the Impact of Different DNMT3A Mutations on Outcome in Younger Adults With Acute Myeloid Leukemia. *J Clin Oncol,* **33,** 2072-2083.

Ge, X.Q., Jackson, D.A. & Blow, J.J. (2007) Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress. *Genes Dev,* **21,** 3331-3341.

Genovese, G., Kahler, A.K., Handsaker, R.E., Lindberg, J., Rose, S.A., Bakhoum, S.F., Chambert, K., Mick, E., Neale, B.M., Fromer, M., Purcell, S.M., Svantesson, O., Landen, M., Hoglund, M., Lehmann, S., Gabriel, S.B., Moran, J.L., Lander, E.S., Sullivan, P.F., Sklar, P., Gronberg, H., Hultman, C.M. & McCarroll, S.A. (2014) Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med,* **371,** 2477-2487.

Gilbert, D.M. (2007) Replication origin plasticity, Taylor-made: inhibition vs recruitment of origins under conditions of replication stress. *Chromosoma,* **116,** 341-347.

Gineau, L., Cognet, C., Kara, N., Lach, F.P., Dunne, J., Veturi, U., Picard, C., Trouillet, C., Eidenschenk, C., Aoufouchi, S., Alcais, A., Smith, O., Geissmann, F., Feighery, C., Abel, L., Smogorzewska, A., Stillman, B., Vivier, E., Casanova, J.L. & Jouanguy, E. (2012) Partial MCM4 deficiency in patients with growth retardation, adrenal insufficiency, and natural killer cell deficiency. *J Clin Invest,* **122,** 821-832.

Gonzalez, M.A., Pinder, S.E., Callagy, G., Vowler, S.L., Morris, L.S., Bird, K., Bell, J.A., Laskey, R.A. & Coleman, N. (2003) Minichromosome maintenance protein 2 is a strong independent prognostic marker in breast cancer. *J Clin Oncol,* **21,** 4306-4313.

Gonzalez, M.A., Tachibana, K.E., Chin, S.F., Callagy, G., Madine, M.A., Vowler, S.L., Pinder, S.E., Laskey, R.A. & Coleman, N. (2004) Geminin predicts adverse clinical outcome in breast cancer by reflecting cell-cycle progression. *J Pathol,* **204,** 121-130.

Gonzalez, M.A., Tachibana, K.E., Adams, D.J., van der Weyden, L., Hemberger, M., Coleman, N., Bradley, A. & Laskey, R.A. (2006a) Geminin is essential to prevent endoreduplication and to form pluripotent cells during mammalian development. *Genes Dev,* **20,** 1880-1884.

Gonzalez, S., Klatt, P., Delgado, S., Conde, E., Lopez-Rios, F., Sanchez-Cespedes, M., Mendez, J., Antequera, F. & Serrano, M. (2006b) Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. *Nature,* **440,** 702-706.

Green, C.L., Evans, C.M., Hills, R.K., Burnett, A.K., Linch, D.C. & Gale, R.E. (2010a) The prognostic significance of IDH1 mutations in younger adult patients with acute myeloid leukemia is dependent on FLT3/ITD status. *Blood,* **116,** 2779-2782.

Green, C.L., Koo, K.K., Hills, R.K., Burnett, A.K., Linch, D.C. & Gale, R.E. (2010b) Prognostic significance of CEBPA mutations in a large cohort of younger adult patients with acute myeloid leukemia: impact of double CEBPA mutations and the interaction with FLT3 and NPM1 mutations. *J Clin Oncol,* **28,** 2739-2747.

Green, C.L., Evans, C.M., Zhao, L., Hills, R.K., Burnett, A.K., Linch, D.C. & Gale, R.E. (2011) The prognostic significance of IDH2 mutations in AML depends on the location of the mutation. *Blood,* **118,** 409-412.

Griffith, J., Black, J., Faerman, C., Swenson, L., Wynn, M., Lu, F., Lippke, J. & Saxena, K. (2004) The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. *Molecular cell,* **13,** 169-178.

Grimwade, D., Walker, H., Oliver, F., Wheatley, K., Harrison, C., Harrison, G., Rees, J., Hann, I., Stevens, R., Burnett, A. & Goldstone, A. (1998) The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood,* **92,** 2322-2333.

Grimwade, D., Hills, R.K., Moorman, A.V., Walker, H., Chatters, S., Goldstone, A.H., Wheatley, K., Harrison, C.J. & Burnett, A.K. (2010) Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood,* **116,** 354-365.

Grimwade, D. & Freeman, S.D. (2014) Defining minimal residual disease in acute myeloid leukemia: which platforms are ready for "prime time"? *Blood,* **124,** 3345-3355.

Grisendi, S., Mecucci, C., Falini, B. & Pandolfi, P.P. (2006) Nucleophosmin and cancer. *Nat Rev Cancer,* **6,** 493-505.

Guerci, A., Pierrez, J., Kohler, F. & Guerci, O. (1989) Prognostic significance of peripheral blood S + G2/M phase size in adult acute non-lymphoblastic leukaemia. *Eur J Haematol,* **43,** 15-21.

Guernsey, D.L., Matsuoka, M., Jiang, H., Evans, S., Macgillivray, C., Nightingale, M., Perry, S., Ferguson, M., LeBlanc, M., Paquette, J., Patry, L., Rideout, A.L., Thomas, A., Orr, A., McMaster, C.R., Michaud, J.L., Deal, C., Langlois, S., Superneau, D.W., Parkash, S., Ludman, M., Skidmore, D.L. & Samuels, M.E. (2011) Mutations in origin recognition complex gene ORC4 cause Meier-Gorlin syndrome. *Nat Genet,* **43,** 360-364.

Gunji, H., Kharbanda, S. & Kufe, D. (1991) Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-beta-D-arabinofuranosylcytosine. *Cancer Res,* **51,** 741-743.

Haferlach, C., Dicker, F., Herholz, H., Schnittger, S., Kern, W. & Haferlach, T. (2008) Mutations of the TP53 gene in acute myeloid leukemia are strongly associated with a complex aberrant karyotype. *Leukemia,* **22,** 1539-1541.

Haferlach, C., Mecucci, C., Schnittger, S., Kohlmann, A., Mancini, M., Cuneo, A., Testoni, N., Rege-Cambrin, G., Santucci, A., Vignetti, M., Fazi, P., Martelli, M.P., Haferlach, T. & Falini, B. (2009) AML with mutated NPM1 carrying a normal or aberrant karyotype show overlapping biologic, pathologic, immunophenotypic, and prognostic features. *Blood,* **114,** 3024-3032.

Hahn, C.N., Chong, C.E., Carmichael, C.L., Wilkins, E.J., Brautigan, P.J., Li, X.C., Babic, M., Lin, M., Carmagnac, A., Lee, Y.K., Kok, C.H., Gagliardi, L., Friend, K.L., Ekert, P.G., Butcher, C.M., Brown, A.L., Lewis, I.D., To, L.B., Timms, A.E., Storek, J., Moore, S., Altree, M., Escher, R., Bardy, P.G., Suthers, G.K., D'Andrea, R.J., Horwitz, M.S. & Scott, H.S. (2011) Heritable GATA2 mutations associated with familial myelodysplastic syndrome and acute myeloid leukemia. *Nature genetics,* **43,** 1012-1017.

Hara, K., Nakayama, K.I. & Nakayama, K. (2006) Geminin is essential for the development of preimplantation mouse embryos. *Genes Cells,* **11,** 1281-1293.

Hart, J.S., George, S.L., Frei, E., 3rd, Bodey, G.P., Nickerson, R.C. & Freireich, E.J. (1977) Prognotic significance of pretreatment proliferative activity in adult acute leukemia. *Cancer,* **39,** 1603-1617.

Hashimoto, K., Araki, K., Osaki, M., Nakamura, H., Tomita, K., Shimizu, E. & Ito, H. (2004) MCM2 and Ki-67 expression in human lung adenocarcinoma: prognostic implications. *Pathobiology,* **71,** 193-200.

Heckl, D., Kowalczyk, M.S., Yudovich, D., Belizaire, R., Puram, R.V., McConkey, M.E., Thielke, A., Aster, J.C., Regev, A. & Ebert, B.L. (2014) Generation of mouse models

of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. *Nat Biotechnol,* **32,** 941-946.

Hemerly, A.S., Prasanth, S.G., Siddiqui, K. & Stillman, B. (2009) Orc1 controls centriole and centrosome copy number in human cells. *Science,* **323,** 789-793.

Hiddemann, W., Buchner, T., Andreeff, M., Wormann, B., Melamed, M.R. & Clarkson, B.D. (1982a) Cell kinetics in acute leukemia: a critical reevaluation based on new data. *Cancer,* **50,** 250-258.

Hiddemann, W., Buchner, T., Andreeff, M., Wormann, B., Melamed, M.R. & Clarkson, B.D. (1982b) Bone marrow biopsy instead of 'marrow juice' for cell kinetic analysis. Comparison of bone marrow biopsy and aspiration material. *Leukemia research,* **6,** 601-612.

Hillen, H., Wessels, J. & Haanen, C. (1975) Bone-marrow-profileration patterns in acute myeloblastic leukaemia determined by pulse cytophotometry. *Lancet,* **1,** 609-611.

Hills, R.K. & Burnett, A.K. (2011) Applicability of a "Pick a Winner" trial design to acute myeloid leukemia. *Blood,* **118,** 2389-2394.

Hills, R.K., Castaigne, S., Appelbaum, F.R., Delaunay, J., Petersdorf, S., Othus, M., Estey, E.H., Dombret, H., Chevret, S., Ifrah, N., Cahn, J.Y., Recher, C., Chilton, L., Moorman, A.V. & Burnett, A.K. (2014) Addition of gemtuzumab ozogamicin to induction chemotherapy in adult patients with acute myeloid leukaemia: a meta-analysis of individual patient data from randomised controlled trials. *Lancet Oncol,* **15,** 986-996.

Hollink, I.H., Zwaan, C.M., Zimmermann, M., Arentsen-Peters, T.C., Pieters, R., Cloos, J., Kaspers, G.J., de Graaf, S.S., Harbott, J., Creutzig, U., Reinhardt, D., van den Heuvel-Eibrink, M.M. & Thiede, C. (2009) Favorable prognostic impact of NPM1 gene mutations in childhood acute myeloid leukemia, with emphasis on cytogenetically normal AML. *Leukemia,* **23,** 262-270.

Holowiecki, J., Grosicki, S., Giebel, S., Robak, T., Kyrcz-Krzemien, S., Kuliczkowski, K., Skotnicki, A.B., Hellmann, A., Sulek, K., Dmoszynska, A., Kloczko, J., Jedrzejczak, W.W., Zdziarska, B., Warzocha, K., Zawilska, K., Komarnicki, M., Kielbinski, M., Piatkowska-Jakubas, B., Wierzbowska, A., Wach, M. & Haus, O. (2012) Cladribine, but not fludarabine, added to daunorubicin and cytarabine during induction prolongs survival of patients with acute myeloid leukemia: a multicenter, randomized phase III study. *J Clin Oncol,* **30,** 2441-2448.

Hossain, M. & Stillman, B. (2012) Meier-Gorlin syndrome mutations disrupt an Orc1 CDK inhibitory domain and cause centrosome reduplication. *Genes Dev,* **26,** 1797-1810.

Hu, J., McCall, C.M., Ohta, T. & Xiong, Y. (2004) Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in response to DNA damage. *Nat Cell Biol,* **6,** 1003-1009.

Hu, J. & Xiong, Y. (2006) An evolutionarily conserved function of proliferating cell nuclear antigen for Cdt1 degradation by the Cul4-Ddb1 ubiquitin ligase in response to DNA damage. *J Biol Chem,* **281,** 3753-3756.

Huang, H.Y., Huang, W.W., Lin, C.N., Eng, H.L., Li, S.H., Li, C.F., Lu, D., Yu, S.C. & Hsiung, C.Y. (2006) Immunohistochemical expression of p16INK4A, Ki-67, and Mcm2 proteins in gastrointestinal stromal tumors: prognostic implications and correlations with risk stratification of NIH consensus criteria. *Ann Surg Oncol,* **13,** 1633-1644.

Huang, S., Xu, X., Wang, G., Lu, G., Xie, W., Tao, W., Zhang, H., Jiang, Q. & Zhang, C. (2016) DNA replication initiator Cdc6 also regulates ribosomal DNA transcription initiation. *J Cell Sci,* **129,** 1429-1440.

Huggett, M.T., Tudzarova, S., Proctor, I., Loddo, M., Keane, M.G., Stoeber, K., Williams, G.H. & Pereira, S.P. (2016) Cdc7 is a potent anti-cancer target in pancreatic cancer due to abrogation of the DNA origin activation checkpoint. *Oncotarget,* **7,** 18495-18507.

Hughes, C.R., Guasti, L., Meimaridou, E., Chuang, C.H., Schimenti, J.C., King, P.J., Costigan, C., Clark, A.J. & Metherell, L.A. (2012) MCM4 mutation causes adrenal failure, short stature, and natural killer cell deficiency in humans. *J Clin Invest,* **122,** 814-820.

Ibarra, A., Schwob, E. & Mendez, J. (2008) Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. *Proc Natl Acad Sci U S A,* **105,** 8956-8961.

Iida, H., Towatari, M., Tanimoto, M., Morishita, Y., Kodera, Y. & Saito, H. (1997) Overexpression of cyclin E in acute myelogenous leukemia. *Blood,* **90,** 3707-3713.

Ito, K., Turcotte, R., Cui, J., Zimmerman, S.E., Pinho, S., Mizoguchi, T., Arai, F., Runnels, J.M., Alt, C., Teruya-Feldstein, J., Mar, J.C., Singh, R., Suda, T., Lin, C.P., Frenette, P.S. & Ito, K. (2016) Self-renewal of a purified Tie2+ hematopoietic stem cell population relies on mitochondrial clearance. *Science*.

Ivey, A., Hills, R.K., Simpson, M.A., Jovanovic, J.V., Gilkes, A., Grech, A., Patel, Y., Bhudia, N., Farah, H., Mason, J., Wall, K., Akiki, S., Griffiths, M., Solomon, E., McCaughan, F., Linch, D.C., Gale, R.E., Vyas, P., Freeman, S.D., Russell, N., Burnett, A.K., Grimwade, D. & Group, U.K.N.C.R.I.A.W. (2016) Assessment of Minimal Residual Disease in Standard-Risk AML. *The New England journal of medicine,* **374,** 422-433.

Jahns-Streubel, G., Reuter, C., Unterhalt, M., Schleyer, E., Wormann, B., Buchner, T. & Hiddemann, W. (1995) Blast cell proliferative activity and sensitivity to GM-CSF in vitro are associated with early response to TAD-9 induction therapy in acute myeloid leukemia. *Leukemia,* **9,** 1857-1863.

Jahns-Streubel, G., Reuter, C., Auf der Landwehr, U., Unterhalt, M., Schleyer, E., Wormann, B., Buchner, T. & Hiddemann, W. (1997) Activity of thymidine kinase and of polymerase alpha as well as activity and gene expression of deoxycytidine deaminase in leukemic blasts are correlated with clinical response in the setting of granulocyte-macrophage colony-stimulating factor-based priming before and during TAD-9 induction therapy in acute myeloid leukemia. *Blood,* **90,** 1968-1976.

Jahns-Streubel, G., Braess, J., Schoch, C., Fonatsch, C., Haase, D., Binder, C., Wormann, B., Buchner, T. & Hiddemann, W. (2001) Cytogenetic subgroups in acute myeloid leukemia differ in proliferative activity and response to GM-CSF. *Leukemia,* **15,** 377-384.

Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P.V., Mar, B.G., Lindsley, R.C., Mermel, C.H., Burtt, N., Chavez, A., Higgins, J.M., Moltchanov, V., Kuo, F.C., Kluk, M.J., Henderson, B., Kinnunen, L., Koistinen, H.A., Ladenvall, C., Getz, G., Correa, A., Banahan, B.F., Gabriel, S., Kathiresan, S., Stringham, H.M., McCarthy, M.I., Boehnke, M., Tuomilehto, J., Haiman, C., Groop, L., Atzmon, G., Wilson, J.G., Neuberg, D., Altshuler, D. & Ebert, B.L. (2014) Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med,* **371,** 2488-2498.

Jordanova, E.S., Corver, W.E., Vonk, M.J., Leers, M.P., Riemersma, S.A., Schuuring, E. & Kluin, P.M. (2003) Flow cytometric sorting of paraffin-embedded tumor tissues considerably improves molecular genetic analysis. *Am J Clin Pathol,* **120,** 327-334.

Juliusson, G., Antunovic, P., Derolf, A., Lehmann, S., Mollgard, L., Stockelberg, D., Tidefelt, U., Wahlin, A. & Hoglund, M. (2009) Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. *Blood,* **113,** 4179-4187.

Kan, Q., Jinno, S., Kobayashi, K., Yamamoto, H. & Okayama, H. (2008) Cdc6 determines utilization of p21(WAF1/CIP1)-dependent damage checkpoint in S phase cells. *J Biol Chem,* **283,** 17864-17872.

Kantarjian, H.M., Thomas, X.G., Dmoszynska, A., Wierzbowska, A., Mazur, G., Mayer, J., Gau, J.P., Chou, W.C., Buckstein, R., Cermak, J., Kuo, C.Y., Oriol, A., Ravandi, F., Faderl, S., Delaunay, J., Lysak, D., Minden, M. & Arthur, C. (2012) Multicenter, randomized, open-label, phase III trial of decitabine versus patient choice, with physician advice, of either supportive care or low-dose cytarabine for the treatment of older patients with newly diagnosed acute myeloid leukemia. *J Clin Oncol,* **30,** 2670-2677.

Kayes, O.J., Loddo, M., Patel, N., Patel, P., Minhas, S., Ambler, G., Freeman, A., Wollenschlaeger, A., Ralph, D.J., Stoeber, K. & Williams, G.H. (2009) DNA replication licensing factors and aneuploidy are linked to tumor cell cycle state and clinical outcome in penile carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **15,** 7335-7344.

Kelly, L.M. & Gilliland, D.G. (2002) Genetics of myeloid leukemias. *Annu Rev Genomics Hum Genet,* **3,** 179-198.

Kelly, L.M., Liu, Q., Kutok, J.L., Williams, I.R., Boulton, C.L. & Gilliland, D.G. (2002) FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. *Blood,* **99,** 310-318.

Kiyoi, H., Naoe, T., Nakano, Y., Yokota, S., Minami, S., Miyawaki, S., Asou, N., Kuriyama, K., Jinnai, I., Shimazaki, C., Akiyama, H., Saito, K., Oh, H., Motoji, T., Omoto, E., Saito, H., Ohno, R. & Ueda, R. (1999) Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood,* **93,** 3074-3080.

Klco, J.M., Spencer, D.H., Miller, C.A., Griffith, M., Lamprecht, T.L., O'Laughlin, M., Fronick, C., Magrini, V., Demeter, R.T., Fulton, R.S., Eades, W.C., Link, D.C., Graubert, T.A., Walter, M.J., Mardis, E.R., Dipersio, J.F., Wilson, R.K. & Ley, T.J. (2014) Functional heterogeneity of genetically defined subclones in acute myeloid leukemia. *Cancer Cell,* **25,** 379-392.

Knapper, S., Russell, N., Gilkes, A., Hills, R.K., Gale, R.E., Cavenagh, J.D., Jones, G., Kjeldsen, L., Grunwald, M.R., Thomas, I., Konig, H., Levis, M.J. & Burnett, A.K. (2016) A randomised assessment of adding the kinase inhibitor lestaurtinib to 1st-line chemotherapy for FLT3-mutated AML. *Blood*.

Kode, A., Manavalan, J.S., Mosialou, I., Bhagat, G., Rathinam, C.V., Luo, N., Khiabanian, H., Lee, A., Murty, V.V., Friedman, R., Brum, A., Park, D., Galili, N., Mukherjee, S., Teruya-Feldstein, J., Raza, A., Rabadan, R., Berman, E. & Kousteni, S. (2014) Leukaemogenesis induced by an activating beta-catenin mutation in osteoblasts. *Nature,* **506,** 240-244.

Koltun, E.S., Tsuhako, A.L., Brown, D.S., Aay, N., Arcalas, A., Chan, V., Du, H., Engst, S., Ferguson, K., Franzini, M., Galan, A., Holst, C.R., Huang, P., Kane, B., Kim, M.H., Li, J., Markby, D., Mohan, M., Noson, K., Plonowski, A., Richards, S.J., Robertson, S., Shaw, K., Stott, G., Stout, T.J., Young, J., Yu, P., Zaharia, C.A., Zhang, W., Zhou, P., Nuss, J.M., Xu, W. & Kearney, P.C. (2012) Discovery of XL413, a potent and selective CDC7 inhibitor. *Bioorg Med Chem Lett,* **22,** 3727-3731.

Kottaridis, P.D., Gale, R.E., Frew, M.E., Harrison, G., Langabeer, S.E., Belton, A.A., Walker, H., Wheatley, K., Bowen, D.T., Burnett, A.K., Goldstone, A.H. & Linch, D.C. (2001) The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood,* **98,** 1752-1759.

Kottaridis, P.D., Gale, R.E., Langabeer, S.E., Frew, M.E., Bowen, D.T. & Linch, D.C. (2002) Studies of FLT3 mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of FLT3 mutations in leukemogenesis, minimal residual disease detection, and possible therapy with FLT3 inhibitors. *Blood,* **100,** 2393-2398.

Kottaridis, P.D., Gale, R.E. & Linch, D.C. (2003) Flt3 mutations and leukaemia. *British journal of haematology,* **122,** 523-538.

Krause, D.S. & Scadden, D.T. (2015) A hostel for the hostile: the bone marrow niche in hematologic neoplasms. *Haematologica,* **100,** 1376-1387.

Kronke, J., Bullinger, L., Teleanu, V., Tschurtz, F., Gaidzik, V.I., Kuhn, M.W., Rucker, F.G., Holzmann, K., Paschka, P., Kapp-Schworer, S., Spath, D., Kindler, T., Schittenhelm, M., Krauter, J., Ganser, A., Gohring, G., Schlegelberger, B., Schlenk, R.F., Dohner, H. & Dohner, K. (2013) Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. *Blood,* **122,** 100-108.

Kubota, Y., Mimura, S., Nishimoto, S., Takisawa, H. & Nojima, H. (1995) Identification of the yeast MCM3-related protein as a component of Xenopus DNA replication licensing factor. *Cell,* **81,** 601-609.

Kufe, D.W., Major, P.P., Egan, E.M. & Beardsley, G.P. (1980) Correlation of cytotoxicity with incorporation of ara-C into DNA. *J Biol Chem,* **255,** 8997-8900.

Kulkarni, A.A., Loddo, M., Leo, E., Rashid, M., Eward, K.L., Fanshawe, T.R., Butcher, J., Frost, A., Ledermann, J.A., Williams, G.H. & Stoeber, K. (2007) DNA replication licensing factors and aurora kinases are linked to aneuploidy and clinical outcome in epithelial ovarian carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **13,** 6153-6161.

Kulkarni, A.A., Kingsbury, S.R., Tudzarova, S., Hong, H.K., Loddo, M., Rashid, M., Rodriguez-Acebes, S., Prevost, A.T., Ledermann, J.A., Stoeber, K. & Williams, G.H. (2009) Cdc7 kinase is a predictor of survival and a novel therapeutic target in epithelial ovarian carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **15,** 2417-2425.

Kunnev, D., Rusiniak, M.E., Kudla, A., Freeland, A., Cady, G.K. & Pruitt, S.C. (2010) DNA damage response and tumorigenesis in Mcm2-deficient mice. *Oncogene,* **29,** 3630-3638.

Kuo, A.J., Song, J., Cheung, P., Ishibe-Murakami, S., Yamazoe, S., Chen, J.K., Patel, D.J. & Gozani, O. (2012) The BAH domain of ORC1 links H4K20me2 to DNA replication licensing and Meier-Gorlin syndrome. *Nature,* **484,** 115-119.

Kushwaha, P.P., Rapalli, K.C. & Kumar, S. (2016) Geminin a multi task protein involved in cancer pathophysiology and developmental process: A review. *Biochimie*.

Lambert, S. & Carr, A.M. (2005) Checkpoint responses to replication fork barriers. *Biochimie,* **87,** 591-602.

Lazenby, M., Gilkes, A.F., Marrin, C., Evans, A., Hills, R.K. & Burnett, A.K. (2014) The prognostic relevance of flt3 and npm1 mutations on older patients treated intensively or non-intensively: a study of 1312 patients in the UK NCRI AML16 trial. *Leukemia,* **28,** 1953-1959.

Lee, B.H., Williams, I.R., Anastasiadou, E., Boulton, C.L., Joseph, S.W., Amaral, S.M., Curley, D.P., Duclos, N., Huntly, B.J., Fabbro, D., Griffin, J.D. & Gilliland, D.G. (2005) FLT3 internal tandem duplication mutations induce myeloproliferative or lymphoid disease in a transgenic mouse model. *Oncogene,* **24,** 7882-7892.

Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J., Harris, C.C., Lichti, C.F., Townsend, R.R., Fulton, R.S., Dooling, D.J., Koboldt, D.C., Schmidt, H., Zhang, Q., Osborne, J.R., Lin, L., O'Laughlin, M., McMichael, J.F., Delehaunty, K.D., McGrath, S.D., Fulton, L.A., Magrini, V.J., Vickery, T.L., Hundal, J., Cook, L.L., Conyers, J.J., Swift, G.W., Reed, J.P., Alldredge, P.A., Wylie, T., Walker, J., Kalicki, J., Watson, M.A., Heath, S., Shannon, W.D., Varghese, N., Nagarajan, R., Westervelt, P., Tomasson, M.H., Link, D.C., Graubert, T.A., DiPersio, J.F., Mardis, E.R. & Wilson, R.K. (2010) DNMT3A mutations in acute myeloid leukemia. *N Engl J Med,* **363,** 2424-2433.

Li, X., Zhao, Q., Liao, R., Sun, P. & Wu, X. (2003) The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. *J Biol Chem,* **278,** 30854-30858.

Li, X., Xu, S., Tan, Y. & Chen, J. (2015) The effects of idarubicin versus other anthracyclines for induction therapy of patients with newly diagnosed leukaemia. *Cochrane Database Syst Rev***,** CD010432.

Linch, D.C., Hills, R.K., Burnett, A.K., Khwaja, A. & Gale, R.E. (2014) Impact of FLT3(ITD) mutant allele level on relapse risk in intermediate-risk acute myeloid leukemia. *Blood,* **124,** 273-276.

Lindsley, R.C., Mar, B.G., Mazzola, E., Grauman, P.V., Shareef, S., Allen, S.L., Pigneux, A., Wetzler, M., Stuart, R.K., Erba, H.P., Damon, L.E., Powell, B.L., Lindeman, N., Steensma, D.P., Wadleigh, M., DeAngelo, D.J., Neuberg, D., Stone, R.M. & Ebert, B.L. (2015) Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. *Blood,* **125,** 1367-1376.

Liu, E., Li, X., Yan, F., Zhao, Q. & Wu, X. (2004) Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation. *J Biol Chem,* **279,** 17283-17288.

Loddo, M., Kingsbury, S.R., Rashid, M., Proctor, I., Holt, C., Young, J., El-Sheikh, S., Falzon, M., Eward, K.L., Prevost, T., Sainsbury, R., Stoeber, K. & Williams, G.H. (2009) Cell-

cycle-phase progression analysis identifies unique phenotypes of major prognostic and predictive significance in breast cancer. *British journal of cancer,* **100,** 959-970.

Loeb, K.R., Kostner, H., Firpo, E., Norwood, T., K, D.T., Clurman, B.E. & Roberts, J.M. (2005) A mouse model for cyclin E-dependent genetic instability and tumorigenesis. *Cancer Cell,* **8,** 35-47.

Lowenberg, B., van Putten, W.L., Touw, I.P., Delwel, R. & Santini, V. (1993) Autonomous proliferation of leukemic cells in vitro as a determinant of prognosis in adult acute myeloid leukemia. *The New England journal of medicine,* **328,** 614-619.

Lowenberg, B., Ossenkoppele, G.J., van Putten, W., Schouten, H.C., Graux, C., Ferrant, A., Sonneveld, P., Maertens, J., Jongen-Lavrencic, M., von Lilienfeld-Toal, M., Biemond, B.J., Vellenga, E., van Marwijk Kooy, M., Verdonck, L.F., Beck, J., Dohner, H., Gratwohl, A., Pabst, T., Verhoef, G., Dutch-Belgian Cooperative Trial Group for, H.-O., German, A.M.L.S.G. & Swiss Group for Clinical Cancer Research Collaborative, G. (2009) High-dose daunorubicin in older patients with acute myeloid leukemia. *N Engl J Med,* **361,** 1235-1248.

Lubbert, M., Ruter, B.H., Claus, R., Schmoor, C., Schmid, M., Germing, U., Kuendgen, A., Rethwisch, V., Ganser, A., Platzbecker, U., Galm, O., Brugger, W., Heil, G., Hackanson, B., Deschler, B., Dohner, K., Hagemeijer, A., Wijermans, P.W. & Dohner, H. (2012) A multicenter phase II trial of decitabine as first-line treatment for older patients with acute myeloid leukemia judged unfit for induction chemotherapy. *Haematologica,* **97,** 393-401.

Luskin, M.R., Lee, J.W., Fernandez, H.F., Abdel-Wahab, O., Bennett, J.M., Ketterling, R.P., Lazarus, H.M., Levine, R.L., Litzow, M.R., Paietta, E.M., Patel, J.P., Racevskis, J., Rowe, J.M., Tallman, M.S., Sun, Z. & Luger, S.M. (2016) Benefit of high-dose daunorubicin in AML induction extends across cytogenetic and molecular groups. *Blood,* **127,** 1551-1558.

Machida, Y.J., Hamlin, J.L. & Dutta, A. (2005) Right place, right time, and only once: replication initiation in metazoans. *Cell,* **123,** 13-24.

Machida, Y.J. & Dutta, A. (2007) The APC/C inhibitor, Emi1, is essential for prevention of rereplication. *Genes Dev,* **21,** 184-194.

Madine, M.A., Khoo, C.Y., Mills, A.D. & Laskey, R.A. (1995) MCM3 complex required for cell cycle regulation of DNA replication in vertebrate cells. *Nature,* **375,** 421-424.

Major, P.P., Egan, E.M., Beardsley, G.P., Minden, M.D. & Kufe, D.W. (1981) Lethality of human myeloblasts correlates with the incorporation of arabinofuranosylcytosine into DNA. *Proc Natl Acad Sci U S A,* **78,** 3235-3239.

Major, P.P., Egan, E.M., Herrick, D.J. & Kufe, D.W. (1982) Effect of ARA-C incorporation on deoxyribonucleic acid synthesis in cells. *Biochem Pharmacol,* **31,** 2937-2940.

Marcucci, G., Maharry, K., Wu, Y.Z., Radmacher, M.D., Mrozek, K., Margeson, D., Holland, K.B., Whitman, S.P., Becker, H., Schwind, S., Metzeler, K.H., Powell, B.L., Carter, T.H., Kolitz, J.E., Wetzler, M., Carroll, A.J., Baer, M.R., Caligiuri, M.A., Larson, R.A. & Bloomfield, C.D. (2010) IDH1 and IDH2 gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin Oncol,* **28,** 2348-2355.

Mariano, A.R., Colombo, E., Luzi, L., Martinelli, P., Volorio, S., Bernard, L., Meani, N., Bergomas, R., Alcalay, M. & Pelicci, P.G. (2006) Cytoplasmic localization of NPM in myeloid leukemias is dictated by gain-of-function mutations that create a functional nuclear export signal. *Oncogene,* **25,** 4376-4380.

Matyskiela, M.E., Lu, G., Ito, T., Pagarigan, B., Lu, C.C., Miller, K., Fang, W., Wang, N.Y., Nguyen, D., Houston, J., Carmel, G., Tran, T., Riley, M., Nosaka, L., Lander, G.C., Gaidarova, S., Xu, S., Ruchelman, A.L., Handa, H., Carmichael, J., Daniel, T.O., Cathers, B.E., Lopez-Girona, A. & Chamberlain, P.P. (2016) A novel cereblon modulator recruits GSPT1 to the CRL4(CRBN) ubiquitin ligase. *Nature,* **535,** 252-257.

May, M., Burger, M., Otto, W., Hakenberg, O.W., Wieland, W.F., May, D., Hofstadter, F., Gotz, S., Niessl, N., Fritsche, H.M., Birnkammer, K., Gilfrich, C., Peter, J., Jain, A., Koch, S., Lebentrau, S., Riedmiller, H., Rossler, W., Denzinger, S., Brookman-May,

S. & Gunia, S. (2013) Ki-67, mini-chromosome maintenance 2 protein (MCM2) and geminin have no independent prognostic relevance for cancer-specific survival in surgically treated squamous cell carcinoma of the penis. *BJU Int,* **112,** E383-390.

McGarry, T.J. & Kirschner, M.W. (1998) Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell,* **93,** 1043-1053.

Mead, A.J., Linch, D.C., Hills, R.K., Wheatley, K., Burnett, A.K. & Gale, R.E. (2007) FLT3 tyrosine kinase domain mutations are biologically distinct from and have a significantly more favorable prognosis than FLT3 internal tandem duplications in patients with acute myeloid leukemia. *Blood,* **110,** 1262-1270.

Mead, A.J., Gale, R.E., Hills, R.K., Gupta, M., Young, B.D., Burnett, A.K. & Linch, D.C. (2008a) Conflicting data on the prognostic significance of FLT3/TKD mutations in acute myeloid leukemia might be related to the incidence of biallelic disease. *Blood,* **112,** 444-445; author reply 445.

Mead, A.J., Gale, R.E., Kottaridis, P.D., Matsuda, S., Khwaja, A. & Linch, D.C. (2008b) Acute myeloid leukaemia blast cells with a tyrosine kinase domain mutation of FLT3 are less sensitive to lestaurtinib than those with a FLT3 internal tandem duplication. *British journal of haematology,* **141,** 454-460.

Melixetian, M., Ballabeni, A., Masiero, L., Gasparini, P., Zamponi, R., Bartek, J., Lukas, J. & Helin, K. (2004) Loss of Geminin induces rereplication in the presence of functional p53. *J Cell Biol,* **165,** 473-482.

Meshinchi, S., Alonzo, T.A., Stirewalt, D.L., Zwaan, M., Zimmerman, M., Reinhardt, D., Kaspers, G.J., Heerema, N.A., Gerbing, R., Lange, B.J. & Radich, J.P. (2006) Clinical implications of FLT3 mutations in pediatric AML. *Blood,* **108,** 3654-3661.

Meyer, S.C. & Levine, R.L. (2014) Translational implications of somatic genomics in acute myeloid leukaemia. *The lancet oncology,* **15,** e382-394.

Miraki-Moud, F., Anjos-Afonso, F., Hodby, K.A., Griessinger, E., Rosignoli, G., Lillington, D., Jia, L., Davies, J.K., Cavenagh, J., Smith, M., Oakervee, H., Agrawal, S., Gribben, J.G., Bonnet, D. & Taussig, D.C. (2013) Acute myeloid leukemia does not deplete normal hematopoietic stem cells but induces cytopenias by impeding their differentiation. *Proc Natl Acad Sci U S A,* **110,** 13576-13581.

Montagnoli, A., Valsasina, B., Croci, V., Menichincheri, M., Rainoldi, S., Marchesi, V., Tibolla, M., Tenca, P., Brotherton, D., Albanese, C., Patton, V., Alzani, R., Ciavolella, A., Sola, F., Molinari, A., Volpi, D., Avanzi, N., Fiorentini, F., Cattoni, M., Healy, S., Ballinari, D., Pesenti, E., Isacchi, A., Moll, J., Bensimon, A., Vanotti, E. & Santocanale, C. (2008) A Cdc7 kinase inhibitor restricts initiation of DNA replication and has antitumor activity. *Nat Chem Biol,* **4,** 357-365.

Moreno, I., Martin, G., Bolufer, P., Barragan, E., Rueda, E., Roman, J., Fernandez, P., Leon, P., Mena, A., Cervera, J., Torres, A. & Sanz, M.A. (2003) Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia. *Haematologica,* **88,** 19-24.

Murphy, S.B., Aur, R.J., Simone, J.V., George, S. & Mauer, A.M. (1977) Pretreatment cytokinetic studies in 94 children with acute leukemia. Relationship to other variables at diagnosis and to outcome of standard treatment. *Blood,* **49,** 683-691.

Nakamura, S., Hirano, I., Okinaka, K., Takemura, T., Yokota, D., Ono, T., Shigeno, K., Shibata, K., Fujisawa, S. & Ohnishi, K. (2010) The FOXM1 transcriptional factor promotes the proliferation of leukemia cells through modulation of cell cycle progression in acute myeloid leukemia. *Carcinogenesis,* **31,** 2012-2021.

Nakao, M., Yokota, S., Iwai, T., Kaneko, H., Horiike, S., Kashima, K., Sonoda, Y., Fujimoto, T. & Misawa, S. (1996) Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia,* **10,** 1911-1918.

Nervi, B., Ramirez, P., Rettig, M.P., Uy, G.L., Holt, M.S., Ritchey, J.K., Prior, J.L., Piwnica-Worms, D., Bridger, G., Ley, T.J. & DiPersio, J.F. (2009) Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. *Blood,* **113,** 6206-6214.

Nishitani, H., Sugimoto, N., Roukos, V., Nakanishi, Y., Saijo, M., Obuse, C., Tsurimoto, T., Nakayama, K.I., Nakayama, K., Fujita, M., Lygerou, Z. & Nishimoto, T. (2006) Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis. *EMBO J,* **25,** 1126-1136.

Obermann, E.C., Eward, K.L., Dogan, A., Paul, E.A., Loddo, M., Munson, P., Williams, G.H. & Stoeber, K. (2005a) DNA replication licensing in peripheral B-cell lymphoma. *The Journal of pathology,* **205,** 318-328.

Obermann, E.C., Eward, K.L., Dogan, A., Paul, E.A., Loddo, M., Munson, P., Williams, G.H. & Stoeber, K. (2005b) DNA replication licensing in peripheral B-cell lymphoma. *The Journal of pathology,* **205,** 318-328.

Oehlmann, M., Score, A.J. & Blow, J.J. (2004) The role of Cdc6 in ensuring complete genome licensing and S phase checkpoint activation. *J Cell Biol,* **165,** 181-190.

Ohno, Y., Yasunaga, S., Ohtsubo, M., Mori, S., Tsumura, M., Okada, S., Ohta, T., Ohtani, K., Kobayashi, M. & Takihara, Y. (2010) Hoxb4 transduction down-regulates Geminin protein, providing hematopoietic stem and progenitor cells with proliferation potential. *Proc Natl Acad Sci U S A,* **107,** 21529-21534.

Ohno, Y., Yasunaga, S., Janmohamed, S., Ohtsubo, M., Saeki, K., Kurogi, T., Mihara, K., Iscove, N.N. & Takihara, Y. (2013) Hoxa9 transduction induces hematopoietic stem and progenitor cell activity through direct down-regulation of geminin protein. *PloS one,* **8,** e53161.

Ono, R., Nakajima, H., Ozaki, K., Kumagai, H., Kawashima, T., Taki, T., Kitamura, T., Hayashi, Y. & Nosaka, T. (2005) Dimerization of MLL fusion proteins and FLT3 activation synergize to induce multiple-lineage leukemogenesis. *The Journal of clinical investigation,* **115,** 919-929.

Ostronoff, F., Othus, M., Lazenby, M., Estey, E., Appelbaum, F.R., Evans, A., Godwin, J., Gilkes, A., Kopecky, K.J., Burnett, A., List, A.F., Fang, M., Oehler, V.G., Petersdorf, S.H., Pogosova-Agadjanyan, E.L., Radich, J.P., Willman, C.L., Meshinchi, S. & Stirewalt, D.L. (2015) Prognostic significance of NPM1 mutations in the absence of FLT3-internal tandem duplication in older patients with acute myeloid leukemia: a SWOG and UK National Cancer Research Institute/Medical Research Council report. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **33,** 1157-1164.

Othus, M., Kantarjian, H., Petersdorf, S., Ravandi, F., Godwin, J., Cortes, J., Pierce, S., Erba, H., Faderl, S., Appelbaum, F.R. & Estey, E. (2014) Declining rates of treatment-related mortality in patients with newly diagnosed AML given 'intense' induction regimens: a report from SWOG and MD Anderson. *Leukemia,* **28,** 289-292.

Owen, C., Barnett, M. & Fitzgibbon, J. (2008) Familial myelodysplasia and acute myeloid leukaemia--a review. *British journal of haematology,* **140,** 123-132.

Pabst, T., Eyholzer, M., Fos, J. & Mueller, B.U. (2009) Heterogeneity within AML with CEBPA mutations; only CEBPA double mutations, but not single CEBPA mutations are associated with favourable prognosis. *Br J Cancer,* **100,** 1343-1346.

Palmqvist, L., Argiropoulos, B., Pineault, N., Abramovich, C., Sly, L.M., Krystal, G., Wan, A. & Humphries, R.K. (2006) The Flt3 receptor tyrosine kinase collaborates with NUP98-HOX fusions in acute myeloid leukemia. *Blood,* **108,** 1030-1036.

Pang, B., Qiao, X., Janssen, L., Velds, A., Groothuis, T., Kerkhoven, R., Nieuwland, M., Ovaa, H., Rottenberg, S., van Tellingen, O., Janssen, J., Huijgens, P., Zwart, W. & Neefjes, J. (2013) Drug-induced histone eviction from open chromatin contributes to the chemotherapeutic effects of doxorubicin. *Nat Commun,* **4,** 1908.

Papaemmanuil, E., Gerstung, M., Bullinger, L., Gaidzik, V.I., Paschka, P., Roberts, N.D., Potter, N.E., Heuser, M., Thol, F., Bolli, N., Gundem, G., Van Loo, P., Martincorena, I., Ganly, P., Mudie, L., McLaren, S., O'Meara, S., Raine, K., Jones, D.R., Teague, J.W., Butler, A.P., Greaves, M.F., Ganser, A., Dohner, K., Schlenk, R.F., Dohner, H. & Campbell, P.J. (2016) Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med,* **374,** 2209-2221.

Paschka, P., Schlenk, R.F., Gaidzik, V.I., Habdank, M., Kronke, J., Bullinger, L., Spath, D., Kayser, S., Zucknick, M., Gotze, K., Horst, H.A., Germing, U., Dohner, H. & Dohner, K. (2010) IDH1 and IDH2 mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with NPM1 mutation without FLT3 internal tandem duplication. *J Clin Oncol,* **28,** 3636-3643.

Paul, T.A., Bies, J., Small, D. & Wolff, L. (2010) Signatures of polycomb repression and reduced H3K4 trimethylation are associated with p15INK4b DNA methylation in AML. *Blood,* **115,** 3098-3108.

Perkins, G. & Diffley, J.F. (1998) Nucleotide-dependent prereplicative complex assembly by Cdc6p, a homolog of eukaryotic and prokaryotic clamp-loaders. *Molecular cell,* **2,** 23-32.

Petersdorf, S.H., Kopecky, K.J., Slovak, M., Willman, C., Nevill, T., Brandwein, J., Larson, R.A., Erba, H.P., Stiff, P.J., Stuart, R.K., Walter, R.B., Tallman, M.S., Stenke, L. & Appelbaum, F.R. (2013) A phase 3 study of gemtuzumab ozogamicin during induction and postconsolidation therapy in younger patients with acute myeloid leukemia. *Blood,* **121,** 4854-4860.

Petersen, B.O., Lukas, J., Sorensen, C.S., Bartek, J. & Helin, K. (1999) Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. *EMBO J,* **18,** 396-410.

Peterson, L.F., Yan, M. & Zhang, D.E. (2007) The p21Waf1 pathway is involved in blocking leukemogenesis by the t(8;21) fusion protein AML1-ETO. *Blood,* **109,** 4392-4398.

Ploen, G.G., Nederby, L., Guldberg, P., Hansen, M., Ebbesen, L.H., Jensen, U.B., Hokland, P. & Aggerholm, A. (2014) Persistence of DNMT3A mutations at long-term remission in adult patients with AML. *Br J Haematol,* **167,** 478-486.

Ponziani, V., Gianfaldoni, G., Mannelli, F., Leoni, F., Ciolli, S., Guglielmelli, P., Antonioli, E., Longo, G., Bosi, A. & Vannucchi, A.M. (2006) The size of duplication does not add to the prognostic significance of FLT3 internal tandem duplication in acute myeloid leukemia patients. *Leukemia,* **20,** 2074-2076.

Prasanth, S.G., Prasanth, K.V. & Stillman, B. (2002) Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. *Science,* **297,** 1026-1031.

Pratcorona, M., Brunet, S., Nomdedeu, J., Ribera, J.M., Tormo, M., Duarte, R., Escoda, L., Guardia, R., Queipo de Llano, M.P., Salamero, O., Bargay, J., Pedro, C., Marti, J.M., Torrebadell, M., Diaz-Beya, M., Camos, M., Colomer, D., Hoyos, M., Sierra, J., Esteve, J. & Grupo Cooperativo Para el Estudio y Tratamiento de las Leucemias Agudas, M. (2013) Favorable outcome of patients with acute myeloid leukemia harboring a low-allelic burden FLT3-ITD mutation and concomitant NPM1 mutation: relevance to post-remission therapy. *Blood,* **121,** 2734-2738.

Preisler, H.D., Azarnia, N., Raza, A., Grunwald, H., Vogler, R., Browman, G., Goldberg, J., Chervenick, P., Miller, K., Brennan, J. & et al. (1984) Relationship between the per cent of marrow cells in S phase and the outcome of remission-induction therapy for acute nonlymphocytic leukaemia. *British journal of haematology,* **56,** 399-407.

Pruitt, S.C., Bailey, K.J. & Freeland, A. (2007) Reduced Mcm2 expression results in severe stem/progenitor cell deficiency and cancer. *Stem Cells,* **25,** 3121-3132.

Radmacher, M.D., Marcucci, G., Ruppert, A.S., Mrozek, K., Whitman, S.P., Vardiman, J.W., Paschka, P., Vukosavljevic, T., Baldus, C.D., Kolitz, J.E., Caligiuri, M.A., Larson, R.A., Bloomfield, C.D., Cancer & Leukemia Group, B. (2006) Independent confirmation of a prognostic gene-expression signature in adult acute myeloid leukemia with a normal karyotype: a Cancer and Leukemia Group B study. *Blood,* **108,** 1677-1683.

Ramnath, N., Hernandez, F.J., Tan, D.F., Huberman, J.A., Natarajan, N., Beck, A.F., Hyland, A., Todorov, I.T., Brooks, J.S. & Bepler, G. (2001) MCM2 is an independent predictor of survival in patients with non-small-cell lung cancer. *J Clin Oncol,* **19,** 4259-4266.

Rampal, R., Alkalin, A., Madzo, J., Vasanthakumar, A., Pronier, E., Patel, J., Li, Y., Ahn, J., Abdel-Wahab, O., Shih, A., Lu, C., Ward, P.S., Tsai, J.J., Hricik, T., Tosello, V.,

Tallman, J.E., Zhao, X., Daniels, D., Dai, Q., Ciminio, L., Aifantis, I., He, C., Fuks, F., Tallman, M.S., Ferrando, A., Nimer, S., Paietta, E., Thompson, C.B., Licht, J.D., Mason, C.E., Godley, L.A., Melnick, A., Figueroa, M.E. & Levine, R.L. (2014) DNA hydroxymethylation profiling reveals that WT1 mutations result in loss of TET2 function in acute myeloid leukemia. *Cell Rep,* **9,** 1841-1855.

Raza, A., Maheshwari, Y. & Preisler, H.D. (1987) Differences in cell cycle characteristics among patients with acute nonlymphocytic leukemia. *Blood,* **69,** 1647-1653.

Raza, A., Preisler, H.D., Day, R., Yasin, Z., White, M., Lykins, J., Kukla, C., Barcos, M., Bennett, J., Browman, G. & et al. (1990) Direct relationship between remission duration in acute myeloid leukemia and cell cycle kinetics: a leukemia intergroup study. *Blood,* **76,** 2191-2197.

Raza, A., Preisler, H., Lampkin, B., Yousuf, N., Tucker, C., Peters, N., White, M., Kukla, C., Gartside, P., Siegrist, C. & et al. (1991) Biological significance of cell cycle kinetics in 128 standard risk newly diagnosed patients with acute myelocytic leukemia. *British journal of haematology,* **79,** 33-39.

Renner, A.G., Dos Santos, C., Recher, C., Bailly, C., Creancier, L., Kruczynski, A., Payrastre, B. & Manenti, S. (2009) Polo-like kinase 1 is overexpressed in acute myeloid leukemia and its inhibition preferentially targets the proliferation of leukemic cells. *Blood,* **114,** 659-662.

Riccardi, A., Danova, M., Montecucco, C., Ucci, G., Cassano, E., Giordano, M., Mazzini, G. & Giordano, P. (1986) Adult acute non-lymphoblastic leukaemia: reliability and prognostic significance of pretreatment bone marrow S-phase size determined by flow cytofluorometry. *Scand J Haematol,* **36,** 11-17.

Rieger, M.A., Hoppe, P.S., Smejkal, B.M., Eitelhuber, A.C. & Schroeder, T. (2009) Hematopoietic cytokines can instruct lineage choice. *Science,* **325,** 217-218.

Rodriguez-Acebes, S., Proctor, I., Loddo, M., Wollenschlaeger, A., Rashid, M., Falzon, M., Prevost, A.T., Sainsbury, R., Stoeber, K. & Williams, G.H. (2010) Targeting DNA replication before it starts: Cdc7 as a therapeutic target in p53-mutant breast cancers. *The American journal of pathology,* **177,** 2034-2045.

Rubnitz, J.E. & Inaba, H. (2012) Childhood acute myeloid leukaemia. *Br J Haematol,* **159,** 259-276.

Rucker, F.G., Schlenk, R.F., Bullinger, L., Kayser, S., Teleanu, V., Kett, H., Habdank, M., Kugler, C.M., Holzmann, K., Gaidzik, V.I., Paschka, P., Held, G., von Lilienfeld-Toal, M., Lubbert, M., Frohling, S., Zenz, T., Krauter, J., Schlegelberger, B., Ganser, A., Lichter, P., Dohner, K. & Dohner, H. (2012) TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. *Blood,* **119,** 2114-2121.

Saha, P., Chen, J., Thome, K.C., Lawlis, S.J., Hou, Z.H., Hendricks, M., Parvin, J.D. & Dutta, A. (1998) Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. *Mol Cell Biol,* **18,** 2758-2767.

Sanz, M.A. & Lo-Coco, F. (2011) Modern approaches to treating acute promyelocytic leukemia. *J Clin Oncol,* **29,** 495-503.

Sasi, N.K., Tiwari, K., Soon, F.F., Bonte, D., Wang, T., Melcher, K., Xu, H.E. & Weinreich, M. (2014) The potent Cdc7-Dbf4 (DDK) kinase inhibitor XL413 has limited activity in many cancer cell lines and discovery of potential new DDK inhibitor scaffolds. *PloS one,* **9,** e113300.

Schessl, C., Rawat, V.P., Cusan, M., Deshpande, A., Kohl, T.M., Rosten, P.M., Spiekermann, K., Humphries, R.K., Schnittger, S., Kern, W., Hiddemann, W., Quintanilla-Martinez, L., Bohlander, S.K., Feuring-Buske, M. & Buske, C. (2005) The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. *The Journal of clinical investigation,* **115,** 2159-2168.

Schlenk, R.F., Dohner, K., Krauter, J., Frohling, S., Corbacioglu, A., Bullinger, L., Habdank, M., Spath, D., Morgan, M., Benner, A., Schlegelberger, B., Heil, G., Ganser, A. & Dohner, H. (2008) Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *The New England journal of medicine,* **358,** 1909-1918.

Schneider, F., Hoster, E., Unterhalt, M., Schneider, S., Dufour, A., Benthaus, T., Mellert, G., Zellmeier, E., Bohlander, S.K., Feuring-Buske, M., Buske, C., Braess, J., Fritsch, S., Heinecke, A., Sauerland, M.C., Berdel, W.E., Buechner, T., Woermann, B.J., Hiddemann, W. & Spiekermann, K. (2009) NPM1 but not FLT3-ITD mutations predict early blast cell clearance and CR rate in patients with normal karyotype AML (NK-AML) or high-risk myelodysplastic syndrome (MDS). *Blood,* **113,** 5250-5253.

Schnittger, S., Schoch, C., Dugas, M., Kern, W., Staib, P., Wuchter, C., Loffler, H., Sauerland, C.M., Serve, H., Buchner, T., Haferlach, T. & Hiddemann, W. (2002) Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood,* **100,** 59-66.

Schnittger, S., Schoch, C., Kern, W., Mecucci, C., Tschulik, C., Martelli, M.F., Haferlach, T., Hiddemann, W. & Falini, B. (2005) Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. *Blood,* **106,** 3733-3739.

Schnittger, S., Bacher, U., Kern, W., Alpermann, T., Haferlach, C. & Haferlach, T. (2011) Prognostic impact of FLT3-ITD load in NPM1 mutated acute myeloid leukemia. *Leukemia,* **25,** 1297-1304.

Scrucca, L., Santucci, A. & Aversa, F. (2007) Competing risk analysis using R: an easy guide for clinicians. *Bone Marrow Transplant,* **40,** 381-387.

Selim, D., Alonzo, T.A., Othus, M., Gerbing, R., Ostronoff, F., Tarlock, K., Kutny, M., Aplenc, R., Kolb, E.A., Radich, J., F.R., A., Gamis, A., Stirewalt, D.L. & Meshinchi, S. (2016) Genomic Subtypes of Nucleophosmin (NPM1) Mutations Are Associated with Clinical Outcome in AML - a COG and SWOG Intergroup Collaboration. *Blood,* **128,** 285.

Sellar, R.S., Fraser, L., Khwaja, A., Gale, R.E., Marafioti, T., Akarca, A., Hubank, M., Brooks, T., Stoeber, K., Williams, G. & Linch, D.C. (2016) Cell cycle status in AML blast cells from peripheral blood, bone marrow aspirates and trephines and implications for biological studies and treatment. *British journal of haematology,* **174,** 275-279.

Senga, T., Sivaprasad, U., Zhu, W., Park, J.H., Arias, E.E., Walter, J.C. & Dutta, A. (2006) PCNA is a cofactor for Cdt1 degradation by CUL4/DDB1-mediated N-terminal ubiquitination. *J Biol Chem,* **281,** 6246-6252.

Seo, J., Chung, Y.S., Sharma, G.G., Moon, E., Burack, W.R., Pandita, T.K. & Choi, K. (2005) Cdt1 transgenic mice develop lymphoblastic lymphoma in the absence of p53. *Oncogene,* **24,** 8176-8186.

Seo, S. & Kroll, K.L. (2006) Geminin's double life: chromatin connections that regulate transcription at the transition from proliferation to differentiation. *Cell Cycle,* **5,** 374-379.

Shima, N., Alcaraz, A., Liachko, I., Buske, T.R., Andrews, C.A., Munroe, R.J., Hartford, S.A., Tye, B.K. & Schimenti, J.C. (2007) A viable allele of Mcm4 causes chromosome instability and mammary adenocarcinomas in mice. *Nat Genet,* **39,** 93-98.

Shinnick, K.M., Eklund, E.A. & McGarry, T.J. (2010) Geminin deletion from hematopoietic cells causes anemia and thrombocytosis in mice. *J Clin Invest,* **120,** 4303-4315.

Shivarov, V. & Bullinger, L. (2014) Expression profiling of leukemia patients: key lessons and future directions. *Exp Hematol,* **42,** 651-660.

Shomori, K., Nishihara, K., Tamura, T., Tatebe, S., Horie, Y., Nosaka, K., Haruki, T., Hamamoto, Y., Shiomi, T., Nakabayashi, M. & Ito, H. (2010) Geminin, Ki67, and minichromosome maintenance 2 in gastric hyperplastic polyps, adenomas, and intestinal-type carcinomas: pathobiological significance. *Gastric Cancer,* **13,** 177-185.

Shreeram, S., Sparks, A., Lane, D.P. & Blow, J.J. (2002) Cell type-specific responses of human cells to inhibition of replication licensing. *Oncogene,* **21,** 6624-6632.

Shrestha, P., Saito, T., Hama, S., Arifin, M.T., Kajiwara, Y., Yamasaki, F., Hidaka, T., Sugiyama, K. & Kurisu, K. (2007) Geminin: a good prognostic factor in high-grade astrocytic brain tumors. *Cancer,* **109,** 949-956.

Slovak, M.L., Kopecky, K.J., Cassileth, P.A., Harrington, D.H., Theil, K.S., Mohamed, A., Paietta, E., Willman, C.L., Head, D.R., Rowe, J.M., Forman, S.J. & Appelbaum, F.R.

(2000) Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. *Blood,* **96,** 4075-4083.

Small, D. (2006) FLT3 mutations: biology and treatment. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program***,** 178-184.

Smith, M.L., Hills, R.K. & Grimwade, D. (2011) Independent prognostic variables in acute myeloid leukaemia. *Blood Rev,* **25,** 39-51.

Sohal, J., Phan, V.T., Chan, P.V., Davis, E.M., Patel, B., Kelly, L.M., Abrams, T.J., O'Farrell, A.M., Gilliland, D.G., Le Beau, M.M. & Kogan, S.C. (2003) A model of APL with FLT3 mutation is responsive to retinoic acid and a receptor tyrosine kinase inhibitor, SU11657. *Blood,* **101,** 3188-3197.

Sportoletti, P., Grisendi, S., Majid, S.M., Cheng, K., Clohessy, J.G., Viale, A., Teruya-Feldstein, J. & Pandolfi, P.P. (2008) Npm1 is a haploinsufficient suppressor of myeloid and lymphoid malignancies in the mouse. *Blood,* **111,** 3859-3862.

Spruck, C.H., Won, K.A. & Reed, S.I. (1999) Deregulated cyclin E induces chromosome instability. *Nature,* **401,** 297-300.

Stiff, T., Alagoz, M., Alcantara, D., Outwin, E., Brunner, H.G., Bongers, E.M., O'Driscoll, M. & Jeggo, P.A. (2013) Deficiency in origin licensing proteins impairs cilia formation: implications for the aetiology of Meier-Gorlin syndrome. *PLoS Genet,* **9,** e1003360.

Stirewalt, D.L., Kopecky, K.J., Meshinchi, S., Appelbaum, F.R., Slovak, M.L., Willman, C.L. & Radich, J.P. (2001) FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood,* **97,** 3589-3595.

Stirewalt, D.L. & Radich, J.P. (2003) The role of FLT3 in haematopoietic malignancies. *Nat Rev Cancer,* **3,** 650-665.

Stirewalt, D.L., Kopecky, K.J., Meshinchi, S., Engel, J.H., Pogosova-Agadjanyan, E.L., Linsley, J., Slovak, M.L., Willman, C.L. & Radich, J.P. (2006) Size of FLT3 internal tandem duplication has prognostic significance in patients with acute myeloid leukemia. *Blood,* **107,** 3724-3726.

Stoeber, K., Halsall, I., Freeman, A., Swinn, R., Doble, A., Morris, L., Coleman, N., Bullock, N., Laskey, R.A., Hales, C.N. & Williams, G.H. (1999) Immunoassay for urothelial cancers that detects DNA replication protein Mcm5 in urine. *Lancet,* **354,** 1524-1525.

Stoeber, K., Swinn, R., Prevost, A.T., de Clive-Lowe, P., Halsall, I., Dilworth, S.M., Marr, J., Turner, W.H., Bullock, N., Doble, A., Hales, C.N. & Williams, G.H. (2002) Diagnosis of genito-urinary tract cancer by detection of minichromosome maintenance 5 protein in urine sediments. *J Natl Cancer Inst,* **94,** 1071-1079.

Stone, R.M., Mandrekar, S., Sanford, B.L., Geyer, S., Bloomfield, C.D., Dohner, K., Thiede, C., Marucci, G., Lo Coco, F., Klisovic, R.B., Wei, A., Sierra, J., Sanz, M.A., Brandwein, J.M., de Witte, T., Niederwieser, D., Appelbaum, F.R., Medieros, B.C., Tallman, M.S., Krauter, J., Schlenk, R.F., Ganser, A., Serve, H., Ehninger, G., Amadori, S., Dohner, H. & Larson, R.A. (2015) The Multi-Kinase Inhibitor Midostaurin (M) Prolongs Survival Compared with Placebo (P) in Combination with Daunorubicin (D)/Cytarabine (C) Induction (ind), High-Dose C Consolidation (consol), and As Maintenance (maint) Therapy in Newly Diagnosed Acute Myeloid Leukemia (AML) Patients (pts) Age 18-60 with FLT3 Mutations (muts): An International Prospsective Randomized (rand) P-Controlled Double-Blind Trial (CALGB 10603/RATIFY [Alliance]). *Blood,* **126,** 6.

Stuermer, A., Hoehn, K., Faul, T., Auth, T., Brand, N., Kneissl, M., Putter, V. & Grummt, F. (2007) Mouse pre-replicative complex proteins colocalise and interact with the centrosome. *Eur J Cell Biol,* **86,** 37-50.

Sugimoto, N., Tatsumi, Y., Tsurumi, T., Matsukage, A., Kiyono, T., Nishitani, H. & Fujita, M. (2004) Cdt1 phosphorylation by cyclin A-dependent kinases negatively regulates its function without affecting geminin binding. *J Biol Chem,* **279,** 19691-19697.

Suzuki, S., Kurata, M., Abe, S., Miyazawa, R., Murayama, T., Hidaka, M., Yamamoto, K. & Kitagawa, M. (2012) Overexpression of MCM2 in myelodysplastic syndromes:

association with bone marrow cell apoptosis and peripheral cytopenia. *Experimental and molecular pathology,* **92,** 160-166.

Suzuki, T., Kiyoi, H., Ozeki, K., Tomita, A., Yamaji, S., Suzuki, R., Kodera, Y., Miyawaki, S., Asou, N., Kuriyama, K., Yagasaki, F., Shimazaki, C., Akiyama, H., Nishimura, M., Motoji, T., Shinagawa, K., Takeshita, A., Ueda, R., Kinoshita, T., Emi, N. & Naoe, T. (2005) Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. *Blood,* **106,** 2854-2861.

Tada, S., Li, A., Maiorano, D., Mechali, M. & Blow, J.J. (2001) Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. *Nat Cell Biol,* **3,** 107-113.

Takeda, D.Y., Parvin, J.D. & Dutta, A. (2005) Degradation of Cdt1 during S phase is Skp2-independent and is required for efficient progression of mammalian cells through S phase. *J Biol Chem,* **280,** 23416-23423.

Tanaka, S. & Diffley, J.F. (2002) Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation. *Genes Dev,* **16,** 2639-2649.

Taniguchi, T., Chikatsu, N., Takahashi, S., Fujita, A., Uchimaru, K., Asano, S., Fujita, T. & Motokura, T. (1999) Expression of p16INK4A and p14ARF in hematological malignancies. *Leukemia,* **13,** 1760-1769.

Teer, J.K., Machida, Y.J., Labit, H., Novac, O., Hyrien, O., Marheineke, K., Zannis-Hadjopoulos, M. & Dutta, A. (2006) Proliferating human cells hypomorphic for origin recognition complex 2 and pre-replicative complex formation have a defect in p53 activation and Cdk2 kinase activation. *J Biol Chem,* **281,** 6253-6260.

Thiede, C., Steudel, C., Mohr, B., Schaich, M., Schakel, U., Platzbecker, U., Wermke, M., Bornhauser, M., Ritter, M., Neubauer, A., Ehninger, G. & Illmer, T. (2002) Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood,* **99,** 4326-4335.

Thiede, C., Koch, S., Creutzig, E., Steudel, C., Illmer, T., Schaich, M. & Ehninger, G. (2006) Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood,* **107,** 4011-4020.

Thol, F., Damm, F., Wagner, K., Gohring, G., Schlegelberger, B., Hoelzer, D., Lubbert, M., Heit, W., Kanz, L., Schlimok, G., Raghavachar, A., Fiedler, W., Kirchner, H., Heil, G., Heuser, M., Krauter, J. & Ganser, A. (2010) Prognostic impact of IDH2 mutations in cytogenetically normal acute myeloid leukemia. *Blood,* **116,** 614-616.

Thol, F., Damm, F., Ludeking, A., Winschel, C., Wagner, K., Morgan, M., Yun, H., Gohring, G., Schlegelberger, B., Hoelzer, D., Lubbert, M., Kanz, L., Fiedler, W., Kirchner, H., Heil, G., Krauter, J., Ganser, A. & Heuser, M. (2011) Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. *J Clin Oncol,* **29,** 2889-2896.

Tsao, C.C., Geisen, C. & Abraham, R.T. (2004) Interaction between human MCM7 and Rad17 proteins is required for replication checkpoint signaling. *EMBO J,* **23,** 4660-4669.

Tsuji, T., Ficarro, S.B. & Jiang, W. (2006) Essential role of phosphorylation of MCM2 by Cdc7/Dbf4 in the initiation of DNA replication in mammalian cells. *Mol Biol Cell,* **17,** 4459-4472.

Tudzarova, S., Trotter, M.W., Wollenschlaeger, A., Mulvey, C., Godovac-Zimmermann, J., Williams, G.H. & Stoeber, K. (2010) Molecular architecture of the DNA replication origin activation checkpoint. *EMBO J,* **29,** 3381-3394.

Vanotti, E., Amici, R., Bargiotti, A., Berthelsen, J., Bosotti, R., Ciavolella, A., Cirla, A., Cristiani, C., D'Alessio, R., Forte, B., Isacchi, A., Martina, K., Menichincheri, M., Molinari, A., Montagnoli, A., Orsini, P., Pillan, A., Roletto, F., Scolaro, A., Tibolla, M., Valsasina, B., Varasi, M., Volpi, D. & Santocanale, C. (2008) Cdc7 kinase inhibitors: pyrrolopyridinones as potential antitumor agents. 1. Synthesis and structure-activity relationships. *J Med Chem,* **51,** 487-501.

Varma, D., Chandrasekaran, S., Sundin, L.J., Reidy, K.T., Wan, X., Chasse, D.A., Nevis, K.R., DeLuca, J.G., Salmon, E.D. & Cook, J.G. (2012) Recruitment of the human Cdt1

replication licensing protein by the loop domain of Hec1 is required for stable kinetochore-microtubule attachment. *Nat Cell Biol,* **14,** 593-603.

Verhaak, R.G., Goudswaard, C.S., van Putten, W., Bijl, M.A., Sanders, M.A., Hugens, W., Uitterlinden, A.G., Erpelinck, C.A., Delwel, R., Lowenberg, B. & Valk, P.J. (2005) Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood,* **106,** 3747-3754.

Vidriales, M.B., Orfao, A., Lopez-Berges, M.C., Gonzalez, M., Lopez-Macedo, A., Ciudad, J., Lopez, A., Garcia, M.A., Hernandez, J., Borrego, D. & et al. (1995) Prognostic value of S-phase cells in AML patients. *British journal of haematology,* **89,** 342-348.

Walter, D., Hoffmann, S., Komseli, E.S., Rappsilber, J., Gorgoulis, V. & Sorensen, C.S. (2016) SCF(Cyclin F)-dependent degradation of CDC6 suppresses DNA re-replication. *Nat Commun,* **7,** 10530.

Walter, M.J., Shen, D., Ding, L., Shao, J., Koboldt, D.C., Chen, K., Larson, D.E., McLellan, M.D., Dooling, D., Abbott, R., Fulton, R., Magrini, V., Schmidt, H., Kalicki-Veizer, J., O'Laughlin, M., Fan, X., Grillot, M., Witowski, S., Heath, S., Frater, J.L., Eades, W., Tomasson, M., Westervelt, P., DiPersio, J.F., Link, D.C., Mardis, E.R., Ley, T.J., Wilson, R.K. & Graubert, T.A. (2012) Clonal architecture of secondary acute myeloid leukemia. *N Engl J Med,* **366,** 1090-1098.

Walter, R.B., Othus, M., Borthakur, G., Ravandi, F., Cortes, J.E., Pierce, S.A., Appelbaum, F.R., Kantarjian, H.A. & Estey, E.H. (2011) Prediction of early death after induction therapy for newly diagnosed acute myeloid leukemia with pretreatment risk scores: a novel paradigm for treatment assignment. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* **29,** 4417-4423.

Welch, J.S., Ley, T.J., Link, D.C., Miller, C.A., Larson, D.E., Koboldt, D.C., Wartman, L.D., Lamprecht, T.L., Liu, F., Xia, J., Kandoth, C., Fulton, R.S., McLellan, M.D., Dooling, D.J., Wallis, J.W., Chen, K., Harris, C.C., Schmidt, H.K., Kalicki-Veizer, J.M., Lu, C., Zhang, Q., Lin, L., O'Laughlin, M.D., McMichael, J.F., Delehaunty, K.D., Fulton, L.A., Magrini, V.J., McGrath, S.D., Demeter, R.T., Vickery, T.L., Hundal, J., Cook, L.L., Swift, G.W., Reed, J.P., Alldredge, P.A., Wylie, T.N., Walker, J.R., Watson, M.A., Heath, S.E., Shannon, W.D., Varghese, N., Nagarajan, R., Payton, J.E., Baty, J.D., Kulkarni, S., Klco, J.M., Tomasson, M.H., Westervelt, P., Walter, M.J., Graubert, T.A., DiPersio, J.F., Ding, L., Mardis, E.R. & Wilson, R.K. (2012) The origin and evolution of mutations in acute myeloid leukemia. *Cell,* **150,** 264-278.

Wheatley, K., Burnett, A.K., Goldstone, A.H., Gray, R.G., Hann, I.M., Harrison, C.J., Rees, J.K., Stevens, R.F. & Walker, H. (1999) A simple, robust, validated and highly predictive index for the determination of risk-directed therapy in acute myeloid leukaemia derived from the MRC AML 10 trial. United Kingdom Medical Research Council's Adult and Childhood Leukaemia Working Parties. *Br J Haematol,* **107,** 69-79.

Whitman, S.P., Ruppert, A.S., Radmacher, M.D., Mrozek, K., Paschka, P., Langer, C., Baldus, C.D., Wen, J., Racke, F., Powell, B.L., Kolitz, J.E., Larson, R.A., Caligiuri, M.A., Marcucci, G. & Bloomfield, C.D. (2008) FLT3 D835/I836 mutations are associated with poor disease-free survival and a distinct gene-expression signature among younger adults with de novo cytogenetically normal acute myeloid leukemia lacking FLT3 internal tandem duplications. *Blood,* **111,** 1552-1559.

Whitman, S.P., Maharry, K., Radmacher, M.D., Becker, H., Mrozek, K., Margeson, D., Holland, K.B., Wu, Y.Z., Schwind, S., Metzeler, K.H., Wen, J., Baer, M.R., Powell, B.L., Carter, T.H., Kolitz, J.E., Wetzler, M., Moore, J.O., Stone, R.M., Carroll, A.J., Larson, R.A., Caligiuri, M.A., Marcucci, G. & Bloomfield, C.D. (2010) FLT3 internal tandem duplication associates with adverse outcome and gene- and microRNA-expression signatures in patients 60 years of age or older with primary cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *Blood,* **116,** 3622-3626.

Williams, G.H., Romanowski, P., Morris, L., Madine, M., Mills, A.D., Stoeber, K., Marr, J., Laskey, R.A. & Coleman, N. (1998) Improved cervical smear assessment using antibodies against proteins that regulate DNA replication. *Proc Natl Acad Sci U S A,* **95,** 14932-14937.

Williams, G.H., Swinn, R., Prevost, A.T., De Clive-Lowe, P., Halsall, I., Going, J.J., Hales, C.N., Stoeber, K. & Middleton, S.J. (2004) Diagnosis of oesophageal cancer by detection of minichromosome maintenance 5 protein in gastric aspirates. *Br J Cancer,* **91,** 714-719.

Williams, G.H. & Stoeber, K. (2007) Cell cycle markers in clinical oncology. *Current opinion in cell biology,* **19,** 672-679.

Wohlschlegel, J.A., Dwyer, B.T., Dhar, S.K., Cvetic, C., Walter, J.C. & Dutta, A. (2000) Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science,* **290,** 2309-2312.

Woodward, A.M., Gohler, T., Luciani, M.G., Oehlmann, M., Ge, X., Gartner, A., Jackson, D.A. & Blow, J.J. (2006) Excess Mcm2-7 license dormant origins of replication that can be used under conditions of replicative stress. *J Cell Biol,* **173,** 673-683.

Wouters, B.J., Lowenberg, B., Erpelinck-Verschueren, C.A., van Putten, W.L., Valk, P.J. & Delwel, R. (2009) Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. *Blood,* **113,** 3088-3091.

Yamamoto, Y., Kiyoi, H., Nakano, Y., Suzuki, R., Kodera, Y., Miyawaki, S., Asou, N., Kuriyama, K., Yagasaki, F., Shimazaki, C., Akiyama, H., Saito, K., Nishimura, M., Motoji, T., Shinagawa, K., Takeshita, A., Saito, H., Ueda, R., Ohno, R. & Naoe, T. (2001) Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood,* **97,** 2434-2439.

Yamazaki, M., Fujii, S., Murata, Y., Hayashi, R. & Ochiai, A. (2010) High expression level of geminin predicts a poor clinical outcome in salivary gland carcinomas. *Histopathology,* **56,** 883-892.

Yang, J., Ramnath, N., Moysich, K.B., Asch, H.L., Swede, H., Alrawi, S.J., Huberman, J., Geradts, J., Brooks, J.S. & Tan, D. (2006) Prognostic significance of MCM2, Ki-67 and gelsolin in non-small cell lung cancer. *BMC Cancer,* **6,** 203.

Yang, J., Ikezoe, T., Nishioka, C., Tasaka, T., Taniguchi, A., Kuwayama, Y., Komatsu, N., Bandobashi, K., Togitani, K., Koeffler, H.P., Taguchi, H. & Yokoyama, A. (2007) AZD1152, a novel and selective aurora B kinase inhibitor, induces growth arrest, apoptosis, and sensitization for tubulin depolymerizing agent or topoisomerase II inhibitor in human acute leukemia cells in vitro and in vivo. *Blood,* **110,** 2034-2040.

Yang, J., Ikezoe, T., Nishioka, C., Nobumoto, A., Udaka, K. & Yokoyama, A. (2013) CD34(+)/CD38(-) acute myelogenous leukemia cells aberrantly express Aurora kinase A. *Int J Cancer,* **133,** 2706-2719.

Yang, R., Nakamaki, T., Lubbert, M., Said, J., Sakashita, A., Freyaldenhoven, B.S., Spira, S., Huynh, V., Muller, C. & Koeffler, H.P. (1999) Cyclin A1 expression in leukemia and normal hematopoietic cells. *Blood,* **93,** 2067-2074.

Yin, J.A., O'Brien, M.A., Hills, R.K., Daly, S.B., Wheatley, K. & Burnett, A.K. (2012) Minimal residual disease monitoring by quantitative RT-PCR in core binding factor AML allows risk stratification and predicts relapse: results of the United Kingdom MRC AML-15 trial. *Blood,* **120,** 2826-2835.

Yokozawa, T., Towatari, M., Iida, H., Takeyama, K., Tanimoto, M., Kiyoi, H., Motoji, T., Asou, N., Saito, K., Takeuchi, M., Kobayashi, Y., Miyawaki, S., Kodera, Y., Ohno, R., Saito, H. & Naoe, T. (2000) Prognostic significance of the cell cycle inhibitor p27Kip1 in acute myeloid leukemia. *Leukemia,* **14,** 28-33.

Yoshida, S., Yamada, M. & Masaki, S. (1977) Inhibition of DNA polymerase-alpha and -beta of calf thymus by 1-beta-D-arabinofuranosylcytosine-5'-triphosphate. *Biochim Biophys Acta,* **477,** 144-150.

Zhang, L., McGraw, K.L., Sallman, D.A. & List, A.F. (2016) The role of p53 in myelodysplastic syndromes and acute myeloid leukemia: molecular aspects and clinical implications. *Leuk Lymphoma***,** 1-14.

Zhou, C., Martinez, E., Di Marcantonio, D., Solanki-Patel, N., Aghayev, T., Peri, S., Ferraro, F., Skorski, T., Scholl, C., Frohling, S., Balachandran, S., Wiest, D.L. & Sykes, S.M. (2016) JUN is a key transcriptional regulator of the unfolded protein response in acute myeloid leukemia. *Leukemia*.

Zhu, W., Chen, Y. & Dutta, A. (2004) Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint. *Mol Cell Biol,* **24,** 7140-7150.

Zittoun, R., Bouchard, M., Facquet-Danis, J., Percie-du-Sert, M. & Bousser, J. (1975) Prediction of the response to chemotherapy in acute leukemia. *Cancer,* **35,** 507-513.

Zwaan, C.M., Meshinchi, S., Radich, J.P., Veerman, A.J., Huismans, D.R., Munske, L., Podleschny, M., Hahlen, K., Pieters, R., Zimmermann, M., Reinhardt, D., Harbott, J., Creutzig, U., Kaspers, G.J. & Griesinger, F. (2003) FLT3 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. *Blood,* **102,** 2387-2394.

SUPPLEMENTARY MATERIAL

Supplementary table 1. Treatment regimens used for patients in the cohort

Intensive Regimen	Treatment Schedule	UK trials in which used
ADE	Daunorubicin 50 mg/m^2 days 1, 3, and 5 Cytosine Arabinoside 100 mg/m^2 12 hourly days 1-10 (1-8 if course 2) Etoposide 100 mg/m^2 days 1-5	AML15, AML17
ATRA-ATO	ATRA 45 mg/m^2 orally in 2 equal doses per day for up to 60 days as part of course 1 or until remission, at the same dose on days 1-14 and days 29-42 of courses 2-4, and days 1-14 of course 5 In addition to ATRA Course 1: Arsenic trioxide 0.3 mg/kg IV days 1-5, then 0.25 mg/kg twice weekly weeks 2-8 Courses 2-5: Arsenic trioxide 0.3 mg/kg IV days 1-5, then 0.25 mg/kg twice weekly weeks 2-4 In addition patients with presenting WCC of $\geq 10x10^9$/l could receive gemtuzumab ozogamicin as a single infusion (6 mg/m^2) within the first 4 days of course 1.	AML17
ATRA-Ida	ATRA 45 mg/m^2 orally in 2 equal doses per day for up to 60 days as part of course 1 and then on days 1-15 of subsequent courses In addition to ATRA Course 1: Idarubicin 12 mg/m^2 IV on days 2, 4, 6, and 8 Course 2: Idarubicin 5 mg/m^2 IV on days 1-4	AML15, AML17

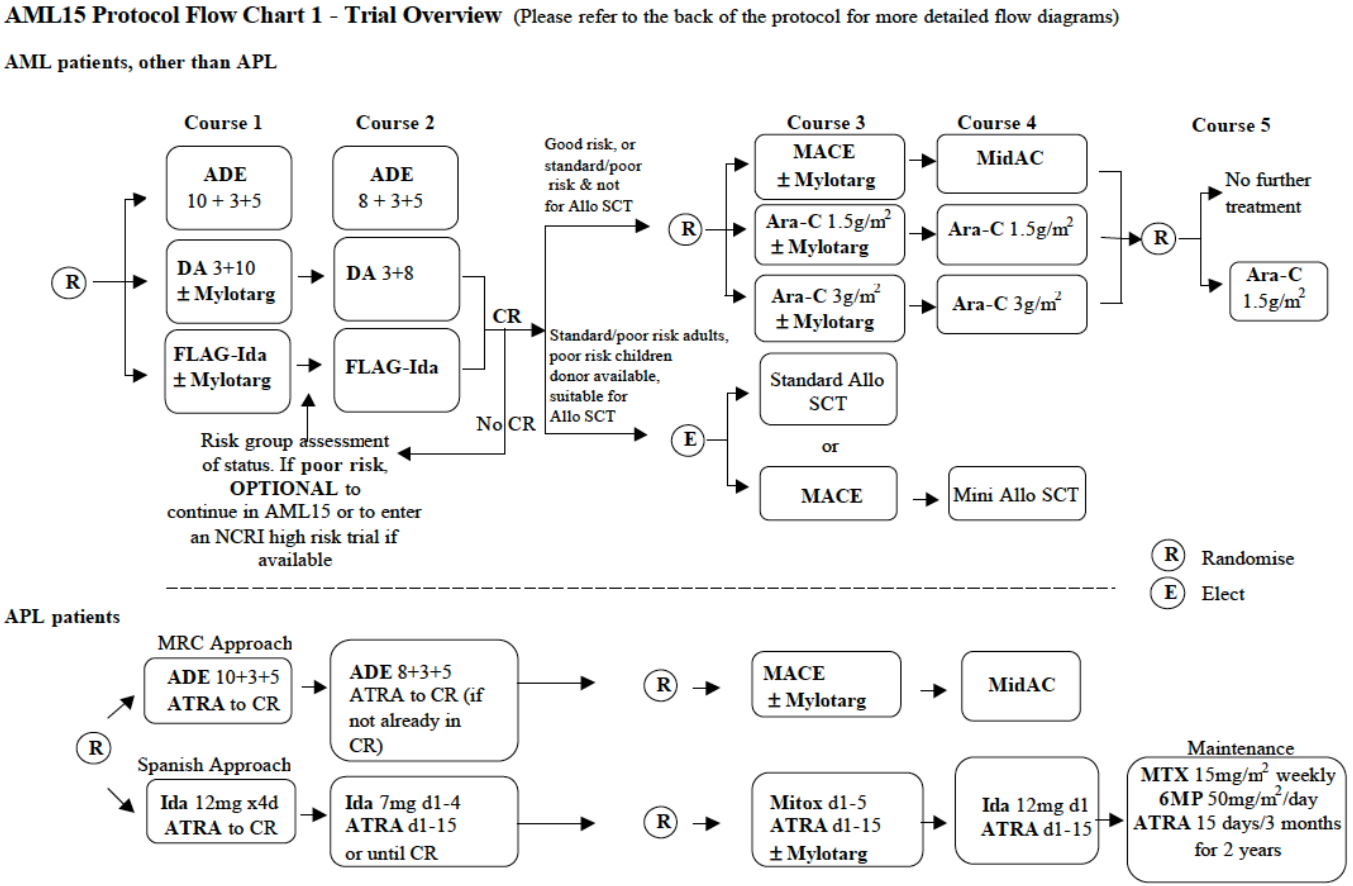
	Course 3: Mitoxantrone 10 mg/m^2 on days 1-4 Course 4: Idarubicin 12 mg/m^2 on day 1	
Azacytidine	75 mg/m^2 per day for 5 days	AML16
Clofarabine	Clofarabine 20 mg/m^2 by IV infusion daily for 5 days	AML16
DA50	Daunorubicin 50 mg/m^2 days 1, 3, and 5 Cytosine Arabinoside 100 mg/m^2 12 hourly days 1-10 (1-8 if course 2)	AML15, AML16
DA60	Daunorubicin 60 mg/m^2 days 1, 3, and 5 Cytosine Arabinoside 100 mg/m^2 12 hourly days 1-10	AML17
DA90	Daunorubicin 90 mg/m^2 days 1, 3, and 5 Cytosine Arabinoside 100 mg/m^2 12 hourly days 1-10	AML17
DAT	Daunorubicin 50 or 35mg/m^2 IV days 1, 2, and 3 IV push Cytosine Arabinoside 100 or 200mg/m^2 IV push 12-hourly on days 1-10 (1-8 if course 2) Thioguanine 100mg/m^2 oral 12-hourly days 1-10 (1-8 if course 2)	AML14
DA 2+5	Daunorubicin 50 mg/m^2 days 1 and 3 Cytosine Arabinoside 100 mg/m^2 12 hourly days 1-5	AML16
DClo	Daunorubicin 50 mg/m^2 days 1, 3, and 5 Clofarabine 20 mg/m^2 days 1-5	AML16, AML17
FLAG-Ida	G-CSF (lenograstim) 263 µg subcutaneous daily on days 1-7 Fludarabine 30 mg/m^2 IV days 2-6 Cytosine Arabinoside 2 grams/m^2 over 4 hours starting 4 hours after fludarabine on days 2-6	AML15, AML17

	Idarubicin 8 mg/m^2 IV daily on days 4-6	
HiDAC1.5	Cytosine Arabinoside 1.5 grams/m^2 IV over 4 hours, 12 hourly on days 1, 3, and 5	AML15
HiDAC3	Cytosine Arabinoside 3 grams/m^2 IV over 4 hours, 12 hourly on days 1, 3, and 5	AML15, AML17
ICE	Idarubicin 10mg/m^2 by slow IV push on days 1 and 3 Cytosine Arabinoside 100mg/m^2 by 2-hour infusion 12-hourly, days 1, 2, and 3 Etoposide 100mg/m^2 by 1-hour infusion daily on days 1, 2, and 3	AML14
LDAC	Cytosine Arabinoside 20 mg by subcutaneous injection twice daily for 10 days	AML14, AML16
MACE	Amsacrine 100 mg/m^2 days 1-5. Cytosine Arabinoside 200 mg/m^2 daily continuous infusion days 1-5 Etoposide 100 mg/m^2 days 1-5	AML15
MidAC (older)	Mitoxantrone 8mg/m^2 IV on days 1, 2, and 3 Cytosine Arabinoside 0.5 grams/m^2 by 2-hour infusion 12-hourly days 1, 2, and 3	AML14
MidAC (younger)	Mitoxantrone 10mg/m^2 daily by slow IV push on days 1-5 inclusive Cytosine Arabinoside 1.0 grams/m^2 by 2-hour IV infusion 12-hourly on days 1-3 inclusive	AML15

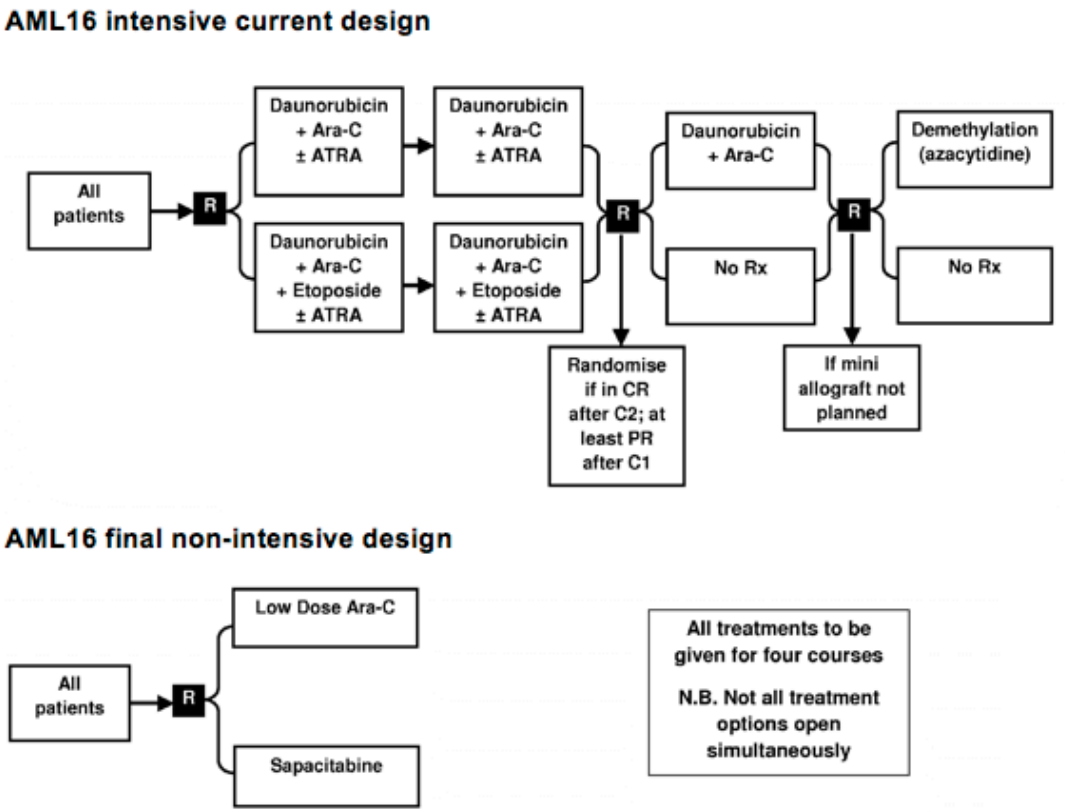
Supplementary table 2. Additional agents used to treat patients in the cohort

Additional Agent	Treatment Schedule	UK trials used in
Arsenic Trioxide	0.25mg/kg days 1-5 and 9-11	AML16
Everolimus	5-10 mg /day from 2 days post cytotoxic chemotherapy to 2 days prior to next course up to a maximum of 28 days	AML17
Gemtuzumab ozogamicin (GO, Mylotarg)	3mg/m^2 IV on day 1 of course (unless otherwise stated)	AML14, AML15, AML16, AML17
Lestaurtinib (CEP-701)	40-80 mg oral twice daily from 2 days post cytotoxic chemotherapy to 2 days prior to next course up to a maximum of 28 days	AML15, AML17
PSC-833	2.0 mg/kg IV loading dose over 2 hours with simultaneous continuous infusion of 10 mg/kg/24 hours for 72 hours, both starting concurrently with the first daunorubicin dose of 35 mg/m^2	AML14
Tipifarnib	300mg bd oral	AML16

Supplementary Figure 1. Example flow chart of AML15 trial



Supplementary Figure 2. Example flow chart of AML16 trial



Supplementary Figure 3. Example flow chart of AML17 trial